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THE ROLE OF ADENOSINE SIGNALING IN MATURE ERYTHROCYTES AND ERYTHROID PROGENITORS

Hong Liu

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**THE ROLE OF ADENOSINE SIGNALING IN MATURE ERYTHROCYTES AND
ERYTHROID PROGENITORS**

A
DISSERTATION

Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

By
Hong Liu
Houston, Texas
May, 2017

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DEDICATION

I dedicate this thesis to my parents, wife, family, mentors and my friends

ACKNOWLEDGEMENTS

The five year experience of my PhD in Houston has been one of the most valuable times during my life so far. I have grown so much both professionally and personally and I could not achieve these all without the support from my amazing advisors, colleagues, friends and family.

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THE ROLE OF ADENOSINE SIGNALING IN MATURE ERYTHROCYTES AND ERYTHROID PROGENITORS

Hong Liu

Advisory Professor: Yang Xia, M.D., Ph.D.

Adenosine is a ubiquitous nucleoside in almost all the cells throughout our bodies. It is highly induced particularly under hypoxia or energy depletion conditions. Adenosine functions as a critical ligand, after binding to membrane-associated adenosine receptors, adenosine initiates a downstream signaling cascade and subsequently contributes to functions of nervous system, immune response, vascular function and even metabolism.

Hypoxia is a condition with limited O₂ availability in the whole body or a region of the body. It is a major consequence of many respiratory and cardiovascular diseases, as well as for people living and working at high altitudes or other low O₂ conditions. Without intervention, it can progress to pulmonary and cerebral edema, stroke, cardiovascular dysfunction and death. Although hypoxia-induced tissue damage is a serious condition with high morbidity and mortality, safe and effective mechanism-based therapies are limited.

The erythrocyte is the most abundant circulating cell type and functions as the only cell-type responsible for O₂ availability in peripheral tissues. Its fundamental

function, O₂ release capacity and O₂ delivery capacity, is determined by hemoglobin-O₂ binding affinity and cell number, respectively.

Although adenosine signaling is beneficial under stress conditions in various organs and tissues via activation of different adenosine receptors, its significance and functional role in the erythroid lineage, particularly under stress conditions, are still unclear.

My dissertation employing multi-disciplinary approaches including human studies, novel genetic tools, and sophisticated pharmacological studies coupled with metabolomic profiling shows for the first time that 1) CD73-dependent elevation of plasma adenosine signaling via erythrocyte specific ADORA2B coupled with AMP-activated protein kinase is a key mechanism for hypoxia adaptation via induction of 2,3-BPG production with O₂ release to counteract severe hypoxia. 2) Adenosine signaling via erythroid ADORA2B is a previously unrecognized purinergic signaling responsible for stress erythropoiesis by regulation of erythroid commitment in a hypoxia-inducible factor 1- α dependent manner.

Thus, my research has identified that adenosine signaling functions as a novel molecular mechanism involved in the regulation of erythrocyte O₂ release capacity and O₂ delivery capacity, providing therapeutic possibilities to enhance O₂ availability and prevent stress-induced tissue damage and inflammation, a strong foundation for future clinical trials to treat diseases associated with hypoxia or hematology disorders provided by my doctoral work.

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Chapter 1: Background introduction

This chapter is based on the review paper “Hong Liu, Yang Xia. Beneficial and detrimental role of adenosine signaling in diseases and therapy. Journal of Applied Physiology Published 27 August 2015 DOI: 10.1152/japplphysiol.00350.2015”

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1.1 Background of adenosine signaling

1.1.1 Metabolism of adenosine

Adenosine is ubiquitously produced in almost all of the cells in our bodies under physiological condition and further produced under hypoxia or energy depletion condition. As a building block and a critical intermediate metabolite of nucleic acids, adenosine is a key signaling molecule that orchestrates the cellular response to hypoxia, energy depletion and tissue damage by activation of its G-protein coupled receptors (GPCR) on multiple cell types¹. Under normal physiological conditions, both extracellular and intracellular adenosine levels are in the nanomolar range. However, under stress conditions, ATP is released from injured cells such as endothelial cells² and neutrophils³ via transmembrane protein channel including pannexins⁴ or connexins^{3, 4}, and subsequently dephosphorylated to extracellular adenosine by ecto-nucleotidases including CD39, which converts ATP to ADP/AMP and CD73, which converts AMP to adenosine^{5, 6}. Under pathological conditions, extracellular adenosine concentrations can reach the millimolar range⁷. Generation of extracellular adenosine through these pathways is the major source of extracellular adenosine production under hypoxia-induced injury. In addition, extracellular adenosine is regulated by adenosine deaminase (ADA), which is responsible for the degradation of extracellular adenosine into inosine⁸. Moreover, extracellular adenosine signaling is terminated by equilibrative nucleoside transporters (ENTs), which are involved in the cellular uptake of adenosine. Once inside the cell, adenosine is metabolized by two enzymes, adenosine kinase (ADK) and adenosine deaminase (ADA). ADA catalyzes the irreversible conversion of adenosine to inosine. ADK phosphorylates adenosine to

AMP, and is critical for regulating intracellular levels of adenosine and maintaining intracellular levels of adenine nucleotides⁷. Intracellular adenosine homeostasis is also maintained by bi-directional equilibrative nucleoside transporters (ENTs) on the plasma membrane, through facilitated diffusion of adenosine in the direction of the concentration gradient (Figure 1)⁹.

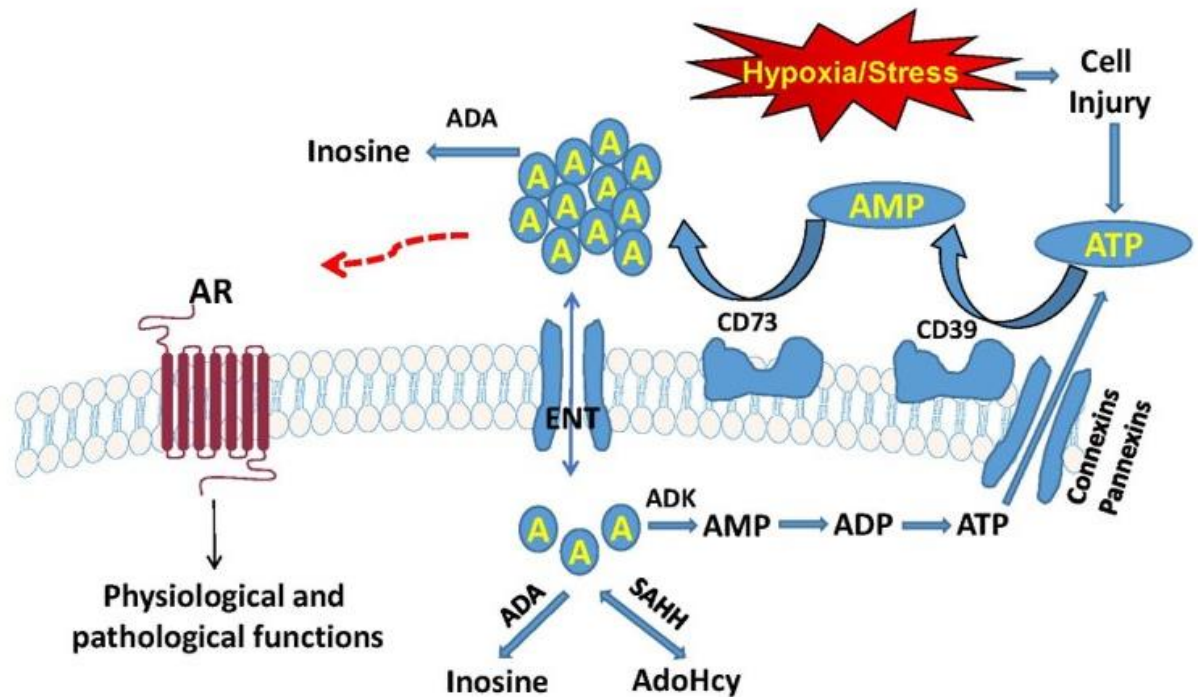


Figure. 1. Metabolism of adenosine signaling.

Cells release ATP through connexins or pannexins channels under hypoxia and other stress conditions. The accumulation of extracellular ATP is dephosphorylated to adenosine (A) by 2 ecto-nucleotidases including CD39 and CD73. Adenosine can further be metabolized by adenosine deaminase (ADA) to inosine or functions as a signaling molecule by activation of its adenosine receptors (AR) on multiple cell types. Once uptake by equilibrative nucleoside transporters (ENTs), adenosine is further metabolized by adenosine kinase (ADK) to AMP, adenosine deaminase (ADA) to inosine, or S-adenosylhomocystine hydrolase (SAHH) to adenosylhomocysteine (AdoHcy).

1.1.2. Adenosine signaling via adenosine receptors

Increases in extracellular adenosine in turn elicit various responses on target cells by engaging cell surface adenosine receptors both in physiological and pathological conditions¹⁰. As GPCRs, adenosine receptors all have a single polypeptide chain which is a structural motif forming seven transmembrane helices. There are four adenosine receptors (ADORA1, ADORA2A, ADORA2B, and ADORA3), and each receptor has a cellular or tissue specific distribution and distinct affinity for adenosine¹¹. ADORA1, ADORA2A, and ADORA3 have a high affinity to extracellular adenosine, while ADORA2B has the lowest affinity to extracellular adenosine. Thus, ADORA2B is normally activated under pathological conditions due to excess accumulation of extracellular adenosine. A1 and A3 adenosine receptors are coupled to adenylyl cyclase by the inhibitory G-protein subunit (G_{ai}) and thereby can lower intracellular levels of the second messenger cyclic adenosine monophosphate (cAMP). In contrast, the A2A and A2B adenosine receptors are commonly coupled to adenylyl cyclase by the stimulatory G-protein subunit (G_{as}) and therefore can induce intracellular cAMP levels. Therefore, signaling through adenosine receptors plays important roles in the regulation of intracellular cAMP and thereby regulate multiple cellular functions including vasodilation in endothelial cells, neurotransmitter release from neuronal cells, neutrophil chemotaxis and vascular smooth muscle cell relaxation^{12, 13} (Figure 2). In addition, other signaling molecules including phospholipase C (PLC), calcium, nitric oxide (NO), reactive oxygen species (ROS), PI3K-AKT, extracellular signal-protein kinase (ERK), and mitogen-activated protein kinases (MAPKs) are implicated functioning downstream of adenosine receptors and subsequently regulating multiple cellular functions. For example, activation of ADORA2A stimulates the PLC pathway

and adenylate cyclase pathway¹⁴. ADORA2A signaling is also engaged in modulation of neutrophil function by regulating production of ROS^{15, 16}. By modulation of NO production via vascular endothelial cells, adenosine through ADORA2A receptor functions as a potent vasodilator involved in tissue blood flow and cellular homeostasis^{17, 18}. In addition, shear stress-mediated elevated adenosine activates ADORA2B, subsequently contributes to endothelial NO synthase phosphorylation via PI3K-AKT, and further generates NO¹⁹. Both pharmacological and genetic studies show that adenosine ADORA2B induces inflammatory cytokine IL-6, and contributes to the renal fibrosis²⁰. The activation of ADORA3 triggers MAPK, and contributes to the critical role of cell growth, survival and differentiation²¹. Other studies report that activation of ADORA3 modulates the proliferation of melanoma cells by regulation of ERK pathway (Figure 2)²². Thus, activation of adenosine receptors are involved in multiple cellular function via multiple downstream signaling cascade.

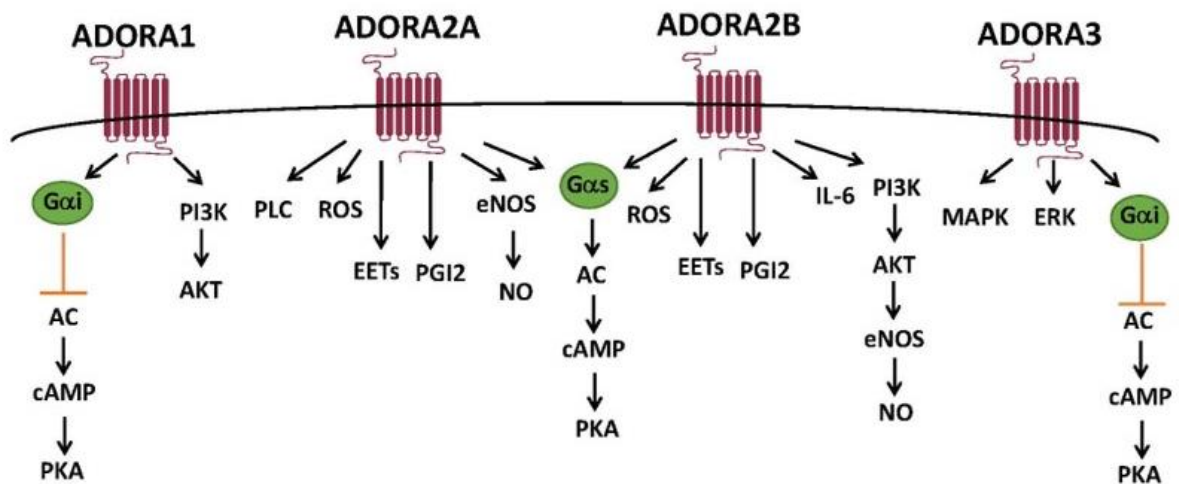


Figure. 2. Adenosine receptor-mediated signaling pathways. Extracellular adenosine functions as signaling molecule by engaging cell surface adenosine receptors (ADORA1, ADORA2A, ADORA2B, and ADORA3). ADORA1 and ADORA3 adenosine receptors are coupled to adenylyl cyclase (AC) by the inhibitory G-protein subunit ($G\alpha_i$) and thereby can lower intracellular levels of the second messenger cyclic adenosine monophosphate (cAMP). In contrast, the ADORA2A and ADORA2B adenosine receptors can induce AC by the stimulatory G-protein subunit ($G\alpha_s$) and therefore can induce intracellular cAMP levels. Activation of both ADORA1 and ADORA2B stimulates phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and activation of both ADORA2A and ADORA2B induces release of ROS, EETs, and PGI₂. PKA, protein kinase A; PLC, phospholipase C; ROS, reactive oxygen species; EETs, epoxyeicosatrienoic acids; PGI₂, prostacyclin; eNOS, endothelial NO synthase; NO, nitric oxide; IL-6, interleukin 6; MAPK, mitogen-activated protein kinases; ERK, extracellular signal-protein kinase.

1.1.3. Adenosine signaling in physiological and pathological conditions

Adenosine is involved in numerous critical physiological processes via activation of its adenosine receptors including modulation of nervous system, immune response, vascular function and metabolism^{13, 23}. Adenosine-mediated biological function is mainly dependent on activation of adenosine receptors, and responses of these cell surface receptors are predominantly determined by adenosine concentrations. Since adenosine levels are generally lower than 1 μ M under physiological condition, most function of adenosine signaling is through activation of A1, A2A or A3 adenosine receptors, which have EC50 values between 0.01 μ M and 1 μ M. In contrast, activation of ADOAR2B requires a higher adenosine concentrations which generally exist under pathophysiological conditions²⁴. With the development and generation of various adenosine receptors agonists or antagonists and four adenosine receptor knockout mouse models, adenosine signaling has been demonstrated as an essential player under pathophysiological conditions by modulation of inflammation, ischemic tissue injury, fibrosis and tissue remodeling²⁵⁻²⁷.

1. 2. Beneficial role of adenosine signaling during acute stage

Recent studies indicate that extracellular adenosine functions as a signaling molecule, which plays an essential role in adaptation to stress especially hypoxia^{26, 28, 29}. Extracellular adenosine is induced during limited oxygen availability or acute injury, and adenosine is critical for hypoxia adaptation, maintenance of cellular function and protection of hypoxia-induced tissue injury. Under acute hypoxic conditions, adenosine plays various beneficial role including vasodilatory effect, anti-vascular endothelial leakage and anti-inflammatory response³⁰⁻³⁴.

1.2.1. Beneficial role in acute heart injury

Beneficial role of adenosine in acute stage was initially found in cardiovascular system showing that adenosine functions as a potent vasodilator increasing blood flow to coronary arteries³⁵. Later on, adenosine was implicated to play a generally protective role in the heart by regulation of heart rate, coronary flow, contraction, inflammatory control and tissue remodeling³⁶. All four adenosine receptors are known to be involved in coronary flow. Generally, adenosine exerts its cardiac electrophysiologic effects mainly through the activation of ADORA1 that leads to a reduction in contraction rate³⁷. Adenosine A2A receptor is the major receptor subtype responsible for coronary blood flow regulation in endothelial-dependent and -independent manner³⁸, previous study reported that adenosine increase coronary flow via vasodilation by promotion of prostacyclin release³⁹. Regadenoson (Lexiscan), a specific ADORA2A agonist, was approved by FDA and utilized for diagnosis of myocardial perfusion imaging⁴⁰. In addition, the Eltzhig group reported that CD73-mediated adenosine signaling via the ADORA2B is important in cardioprotection by ischemic preconditioning. However, Chen and colleagues reported that selective inhibition of adenosine A3 receptor significantly attenuate pressure overload-induced left ventricular hypertrophy and dysfunction⁴¹. These results suggest that selective CD73 agonists and ADORA2B agonists are potential therapeutic possibilities for myocardial ischemia, and specific ADORA3 antagonists may be a novel strategy to counteract pressure overload-induced left ventricular hypertrophy and dysfunction^{41, 42}.

1.2.2. Beneficial role in acute lung injury

Acute lung injury (ALI) is defined as pulmonary edema and severe hypoxia. Multiple factors including pneumonia, aspiration or lung contusion, or indirect injury such as sepsis, severe trauma, blood transfusion cause ALI. Approximately 200,000 patients develop ALI in the US annually. However, due to the lack of understanding the molecular mechanism involved in the development and progression of ALI, no effective therapeutic options are available. Several groups reported that adenosine serves beneficial functions on features of ALI such as enhancing alveolar-capillary barrier function and dampening inflammation, and substantially protects against ALI resulting from hypoxia or ischemia^{43, 44}. Follow-up genetic and pharmacological studies reported that the adenosine mediated beneficial role in ALI is via ADORA2B in a CD73 dependent manner^{45, 46}. Therefore, these studies provide potential development of adenosine-based therapies for the treatment of ALI^{44, 47, 48}.

1.2.3. Beneficial role in acute kidney injury

Acute kidney injury (AKI), characterized as the rapid dysfunction of kidney, is currently the leading cause of mortality and morbidity in hospitalized patients, therefore effective therapeutic strategies are urgently needed. Among multiple factors, renal ischemia is the most common cause of AKI. Previous studies indicated that all four adenosine receptors are expressed in the kidney and are involved in progression of AKI⁴⁹. Particularly, several studies reported adenosine A1 receptor signaling protects the kidney from ischemia-reperfusion injury⁵⁰⁻⁵³. Linder's group showed that adenosine A2A receptor signaling prevents ischemia-induced injury via modulation of inflammatory cells^{54, 55}. In addition, pharmacological and genetic studies demonstrate that adenosine A2B receptor signaling is involved in renal protection during

preconditioning⁵⁶. In contrast, activation of the ADORA3 is implicated as detrimental during renal ischemia⁵².

1.2.4. Beneficial role in brain

As an important signaling molecule, adenosine coupling with its specific receptor functions as an upstream neuromodulator of neurotransmitters involved in the homeostasis and modulation of multiple brain function^{57, 58}. For example, previous studies demonstrated that adenosine is present in the extracellular fluid in brain, its level is dramatically induced in the condition of hypoxia or ischemia, and subsequently plays critical effects through activation of its specific receptors. Although all four adenosine receptors are expressed in the mouse forebrain, ADORA1 and ADORA2A have the highest abundance in the brain. Thus, those two adenosine receptors play critical roles in the brain function, while ADORA2B and ADORA3 have relatively modest impact on brain function^{59, 60}. It is found that ADORA1 is located presynaptically, postsynaptically and nonsynaptically in brain⁵⁹, and mainly underlies effect of adenosine in neuronal circuits by selectively depressing excitatory synaptic transmission⁶¹. Both pharmacological evidence and genetic ADORA1 knockout mouse studies demonstrate neuro-protective role of ADORA1 in ischemia/hypoxia models of brain injury⁶². In contrast, ADORA2A are demonstrated to have a widespread distribution in the brain⁵⁹. Several lines of evidence suggest that adenosine signaling via ADORA2A is neuroprotective under different pathological conditions including hypoxia⁶³, ischemia^{64, 65}, and hypoglycemia⁶⁶. Mechanistic studies demonstrated adenosine-mediated protective effect in the brain is through ADORA2A by controlling brain vascular function through endothelial cells. For instance, ADORA2A plays a beneficial role in preventing brain ischemia by induction of cerebral blood flow (CBF) in

multiple conditions including energy failure, tissue acidosis, imbalance of ion homeostasis and cytotoxic edema^{67, 68}. In addition, Winn and colleagues found that the capacity to modulate CBF in response to hypotension was significantly impaired in ADORA2A knockout mice, and treatment with extracellular adenosine transporter inhibitor, dipyridamole, significantly increases circulating adenosine concentrations and subsequently improves CBF in mice, indicating the importance of adenosine ADORA2A in physiologic vascular regulation of CBF⁶⁹. Furthermore, both pharmacological and genetic studies demonstrated that ADORA2A stimulates proliferation of Schwann cells⁷⁰. Additional studies showed that specific activation of other adenosine receptors contributes to the adenosine-mediated neuroprotective effects as well. For instance, preclinical study showed that A3 specific agonist prevents ischemic brain injury through suppression of apoptosis in wild type mice, but not in the ADORA3-deficient mice⁷¹. Clinical human studies demonstrated that adenosine plays a role of vasodilatation in the cerebral circulation which can be applied for investigation of cerebrovascular perfusion capacity in patients with carotid occlusive disease⁷². Overall, adenosine signaling via its specific receptors plays an important role in brain function and modulating adenosine signaling is likely an effective treatment for brain ischemic injury and damage. **2.5.**

1.2.5 Beneficial role in multiple organ damage at acute stage

Adenosine was reported to be beneficial under stress conditions in various organs and tissues through different adenosine receptors⁷³⁻⁷⁶. Several studies report that adenosine play a otoprotective role in the auditory system to counteract intense noise exposure^{77, 78}. Cronstein's group demonstrate that adenosine A2A signaling plays beneficial role in skin by promoting wound healing and angiogenesis⁷⁶. Colgan and

colleagues used pharmacologic and genetic approaches to show that adenosine signaling via the A2B receptor attenuates tissue injury and inflammation in mucosal organs during intestinal ischemia and colitis⁷⁹⁻⁸². Gnad *et al.* demonstrate that adenosine stimulate brown adipose tissue thermogenesis via ADORA2A, and ADORA2A selective agonist prevent high fat diet-induced obesity in mice⁸³. Linden's group reported that elevated adenosine protects the liver from ischemic reperfusion injury via ADORA2A signaling⁸⁴. In addition, they show that ADORA2A signaling prevents pulmonary inflammation in a sickle cell disease (SCD) mouse model by reducing invariant natural killer cells. Therefore, FDA-approved ADORA2A specific agonist, regadenoson, is currently utilized to conduct a clinical trial in the treatment of patients with SCD^{85, 86}.

Table 1. The beneficial role of adenosine signaling in acute conditions

Organs	Ado receptors	Functions	Cell types
Heart	ADORA1	Slow heart rate	-----
	ADORA2A	Vasodilation	Endothelial cells
	ADORA2B	Ischemic preconditioning	-----
Lung	ADORA2A	Anti-inflammation	Immune cells
	ADORA2B	Vascular barrier function	Endothelial cells
Kidney	ADORA1	Anti-inflammation/apoptosis	Immune cells
	ADORA2A	Anti-inflammation	Immune cells
	ADORA2B	Vascular barrier function	Endothelial cells
Brain	ADORA1	Inhibit excitatory transmission	synapse
	ADORA2A	Increase cerebral blood flow	Endothelial cells
	ADORA3	Anti-apoptosis	-----
Liver	ADORA2A	Anti-inflammation	Immune cells
Skin	ADORA2A	Wound healing	Endothelial cells/Immune cells
Intestine	ADORA2B	Anti-inflammation	Immune cells

1.3 Adenosine signaling in erythrocyte

Although erythrocyte is the most abundant cell type in the body, the role of adenosine in erythrocyte has not been recognized. Recently, our laboratory first reported the functional role of adenosine signaling in erythrocyte in physiology condition. Erythrocyte is the major source of O₂ to each organ and tissue, and its O₂ carrying capacity is mainly regulated by hemoglobin- binding O₂ affinity. Previous studies showed that hemoglobin- binding O₂ affinity is tightly regulated by the 2,3-bisphosphoglycerate (2,3-BPG), which is an important erythroid specific metabolite existing in the glycolytic pathway. However, how the level of 2,3-BPG was regulated is still unclear. By using genetic mouse models, pharmacological tools coupled with human evident, we revealed an important role of adenosine signaling in the regulation of erythrocyte 2,3-BPG. In details, we showed that adenosine specific analogue, 5'-N-ethylcarboxamidoadenosine (NECA), can directly induces 2,3-BPG levels in cultured mouse mature RBCs. Furthermore, by using four adenosine receptor deficient mice models, we showed that NECA can induce 2,3-BPG level in cultured RBCs isolated from WT, Adora1, Adora2a and Adora3 mice, but not in the Adora2b mice, which indicates that adenosine signaling-induced erythrocyte 2,3-BPG in RBC is via ADORA2B. However, what is the significance and functional role of adenosine signaling-mediated regulation of RBC 2,3-BPG particularly in stress condition, and how 2,3-BPG was regulated by adenosine ADORA2B in RBC is still unclear. These important questions will be further addressed in the Chapter 2 of my dissertation.

1.4 High altitude hypoxia

High altitude hypoxia is a condition in which the O₂ availability is limited, therefore O₂ delivery from circulation to peripheral tissues is limited. This directly causes poor exercise performance, even poor walking performance, and it can have substantial impact on overall well-being and cognitive function. Without intervention, high altitude hypoxia induces pulmonary edema, vascular damage and interstitial fibrosis and death. Thus, improving O₂ delivery at the tissue level has the potential to improve peripheral oxygen delivery and thereby raise exercise performance and reverse cognitive deficits caused by hypoxemia. While the relevance of improving O₂ delivery has clear impact on military veterans with hypoxemia secondary to chronic respiratory diseases, high altitude residence can cause similar levels of hypoxemia. Current strategies to counteract hypoxia are limited due to a lack of fundamental understanding of the molecular mechanisms underlying adaptation to hypoxia. The erythrocyte is the most abundant cell type in the circulation and is responsible for delivery of O₂ to peripheral tissues. To function effectively in O₂ uptake, transport and delivery, erythrocytes tightly dependent on sophisticated regulation of Hb-O₂ affinity by endogenous allosteric modulators. One of the best known allosteric modulators is (2,3-BPG), a metabolic byproduct of glycolysis synthesized primarily in erythrocytes for the purpose of regulating Hb-O₂ affinity⁸⁷. It has been known for more than four decades that when humans ascend to high altitude the concentration of 2,3-BPG in erythrocytes increases significantly and its elevation is correlated with an increased capacity of O₂ release from hemoglobin⁸⁸. However, the molecular basis underlying altitude

hypoxia mediated induction of erythrocyte 2,3-BPG remains unknown and the functional role of elevated erythrocyte 2,3-BPG in high altitude adaptation is unclear.

1.5 Erythropoiesis and stress erythropoiesis

Erythropoiesis is a dynamic process to synthesize new erythrocyte, the only cell-type responsible for delivering O₂ to peripheral tissues in our body. Basically, it can be divided into two types of erythropoiesis including primitive erythropoiesis and definitive erythropoiesis. Primitive erythropoiesis is occurred in the fetal life, which included 3 stages based on the duration of pregnancy. The first 2 months called mesoblastic stage which was occurred in yolk sac. The second stage-hepatic stage-was mainly happened in liver, spleen and lymphoid organs. The last stage-myeloid stage-was occurred in red bone marrow. Definitive erythropoiesis is the predominant one in adults, and mainly occurred in bone marrow. Normally, 2.4 million new erythrocytes per second are produced within bone marrow (BM) from erythroid progenitors to enucleated reticulocytes and eventually mature erythrocytes with a 120 day life span in the peripheral circulation. Basal erythropoiesis can be further induced, known as stress erythropoiesis, in response to high altitude, blood loss, infection, BM suppression and anemia to produce more circulating reticulocytes and erythrocytes to counteract insufficient tissue oxygenation^{1,2}. Failure to stimulate effective erythropoiesis, results in hypoxia which can progress to pulmonary edema, stroke, cardiovascular damage, multi-organ injury and death³⁻⁶. Thus, stress-induced erythropoiesis is an important adaptive response for survival under hypoxic conditions. Erythropoiesis is an

extremely dynamic process finely regulated by cytokines, hormones, growth factors, among others at transcriptional and translational levels^{1,2}. Stress-induced erythropoiesis is defined as stimulated basal erythropoiesis with expansion of the erythroid progenitor pool, resulting in reticulocytosis and splenomegaly. Stress erythropoiesis is frequently induced by insufficient tissue oxygenation including high altitude, blood loss, infection, myeloablation, and anemia. Inability to adapt to hypoxia associated with these conditions can result in multi-tissue damage, stroke, cardiovascular dysfunction and even death³⁻⁶. Thus, stress erythropoiesis is an important hypoxic adaptive response for survival. Stress erythropoiesis has been long speculated to be linked with increased metabolic requirements⁷. The energy metabolism of major nutrient including glucose, glutamine and fatty acids is important for the survival, proliferation and differentiation to adapt to physiological and pathological stress conditions. With the development of innovative metabolomic profiling and state of art isotopically labelled metabolic flux approaches⁸, glucose and glutamine metabolism are essential for stress erythropoiesis^{7, 9}.

1.6 Sickle cell disease

Sickle cell disease is an inherited genetic blood disorder with high mortality and morbidity that results from a single mutation in hemoglobin, the protein that carries oxygen in red blood cells throughout our bodies. Although this disease was discovered more than a century ago, we still lack effective mechanism-based therapies. The mutant hemoglobin causes the red blood cells to acquire an unusual sickle shape that hinders movement through blood vessels. The clumps of sickled

cells block blood flow resulting in pain, serious infections, and organ damage. Our laboratory has discovered a process that contributes to enhanced red blood cell sickling, inflammation and progression of the disease and has identified a mechanism-based therapeutic approach to inhibit this process.

Chapter 2 Role of adenosine signaling in mature erythrocyte

This chapter is based on the paper “Hong Liu, Yujin Zhang, Hongyu Wu, Angelo D’Alessandro, Gennady G. Yegutkin, Anren Song, Kaiqi Sun, Jessica Li, Ning-Yuan Cheng, Aji Huang, Yuan Edward Wen, Ting Ting Weng, Fayong Luo, Travis Nemkov, Hong Sun, Rodney E. Kellems, Harry Karmouty-Quintana, Kirk C. Hansen, Bihong Zhao, Andrew W. Subudhi, Sonja Jameson-Van Houten, Colleen G. Julian, Andrew T. Lovering, Holger K. Eltzschig, Michael R. Blackburn, Robert C. Roach, Yang Xia. Beneficial Role of Erythrocyte Adenosine A2B Receptor–Mediated AMP-Activated Protein Kinase Activation in High-Altitude Hypoxia. *Circulation*, Originally published August 1, 2016 2016;134:405-421, <https://doi.org/10.1161/CIRCULATIONAHA.116.021311>”

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2.1 Introduction

High-altitude hypoxia is a challenging condition due to limited O₂ supply. The ability to adjust to high-altitude hypoxia varies considerably among individuals. Failure to adapt to high-altitude hypoxia is associated with poor exercise performance, severe headache, dizziness and vomiting. Without intervention, it can progress to pulmonary and cerebral edema, stroke, cardiovascular dysfunction and death⁸⁹⁻⁹¹. Current strategies to counteract hypoxia are limited due to a lack of fundamental understanding of the molecular mechanisms underlying adaptation to hypoxia.

The erythrocyte is the most abundant circulating cell type and responsible for delivery of O₂ to peripheral tissues. To function effectively in O₂ uptake, transport and delivery, erythrocytes rely on sophisticated regulation of Hb-O₂ affinity by endogenous allosteric modulators. One of the best known allosteric modulators is 2,3-bisphosphoglycerate (2,3-BPG), a metabolic byproduct of glycolysis synthesized primarily in erythrocytes for the purpose of regulating Hb-O₂ affinity⁸⁷. It has been known for more than four decades that when humans ascend to high altitude the concentration of 2,3-BPG in erythrocytes increases significantly and its elevation is correlated with an increased capacity of O₂ release from hemoglobin⁸⁸.

However, the molecular basis underlying altitude hypoxia mediated induction of erythrocyte 2,3-BPG remains unknown and the functional role of elevated erythrocyte 2,3-BPG in high altitude adaptation is unclear.

To identify metabolic alterations of erythrocytes in response to hypoxia, we conducted nonbiased high throughput metabolomic profiling of erythrocytes from humans at sea level and at high altitude. Our results confirmed early studies that erythrocyte 2,3-BPG levels are significantly elevated in response to high altitude, but also led us to discover that circulating adenosine levels and activity of soluble CD73 (sCD73), a key enzyme to generate extracellular adenosine, were significantly enhanced in 21 lowland volunteers within two hours of arrival at 5260 m and continued to increase with time at this elevation. Significantly, hypoxia-induced elevated plasma adenosine level is correlated with erythrocyte 2,3-BPG and O₂ releasing capacity in human subjects. *In vivo* genetic mouse studies coupled with *in vitro* both human and mouse studies demonstrated that increased extracellular adenosine signaling via erythrocyte specific ADORA2B, led to the sequential activation of AMPK and 2,3-BPG mutase, resulting in increased 2,3-BPG production and enhanced O₂ release capacity to peripheral tissues. Thus, our findings highlight the importance of this newly identified erythrocyte adenosine

signaling pathway via AMPK in human adaptation to high altitude and identify several attractive targets to counteract hypoxia in humans.

2.2 Materials and Methods

2.2.1 Human Subjects

This study was conducted as part of the Altitudeomics project examining the integrative physiology of human responses to hypoxia⁹². In brief, all procedures conformed to the Declaration of Helsinki and were approved by the Universities of Colorado and Oregon Institutional Review Boards and the US Department of Defense Human Research Protection office. After written informed consent, recreationally active sea-level habitants participated in the adenosine study (mean \pm SD age, 21 \pm 1 years; stature, 1.78 \pm 0.10 m; body mass, 69 \pm 11 kg; maximum O₂ uptake [Vo₂max, 6.4 \pm 0.2 mL kg⁻¹ min⁻¹]. The participants were non-smokers, free from cardiorespiratory disease, born and raised at <1500 m, and had not travelled to elevations >1000 m in the 3 months prior to investigation.

Each subject was studied near sea level (SL) (130 m, average PB=749 mmHg) and on the first and sixteenth days at Mt Chacaltaya, Bolivia (5260 m; average PB=406 mmHg; AT1, AT16). Baseline studies at SL were conducted over a two-week period in Eugene, OR, USA. Approximately one month after the SL studies, subjects traveled to Bolivia in pairs on successive days. Upon arrival at El Alto (4050m) after an overnight flight, subjects immediately descended to Coroico, Bolivia (1525 m; PB=639 mmHg). Subjects rested for 48 hrs in Coroico to limit the effects of jet lag and were then driven over three hrs to 5260 m. To provide an acute change in inspired PO₂ from 1525 m to 5260 m, subjects breathed supplemental oxygen (2 L/min, nasal cannula or mask) during the drive. On arrival

at 5260 m, the first subject immediately began the experimental protocol as previously described⁹².

2.2.2 Metabolomic Profiling

Metabolomics extraction. RBCs (100 µl) were immediately extracted in ice-cold lysis/extraction buffer (methanol:acetonitrile:water 5:3:2) at 1:9 dilutions. Samples were then agitated at 4°C for 30 min and then centrifuged at 10,000g for 15min at 4°C. Protein and lipid pellets were discarded, while supernatants were stored at -80°C prior to metabolomics analyses⁹³.

Metabolomics analysis. Ten µl of RBC extracts were injected into an UHPLC system (Ultimate 3000, Thermo, San Jose, CA, USA) and run on a Kinetex XB-C18 column (150x2.1 mm i.d., 1.7 µm particle size – Phenomenex, Torrance, CA, USA) using a 3min isocratic gradient at 250 µl/min (mobile phase: 5% acetonitrile, 95% 18 mΩ H₂O, 0.1% formic acid). The UHPLC system was coupled online with a QExactive system (Thermo, San Jose, CA, USA), scanning in Full MS mode (2 µscans) at 70,000 resolution in the 60-900 m/z range, 4kV spray voltage, 15 sheath gas and 5 auxiliary gas, operated in negative and then positive ion mode (separate runs). Calibration was performed before each analysis against positive or negative ion mode calibration mixes (Piercenet – Thermo Fisher, Rockford, IL, USA) to ensure sub ppm error of the intact mass. Metabolite assignments were performed using the software Maven (Princeton, NJ, USA), upon conversion of raw files into mzXML format through MassMatrix (Cleveland, OH, USA). The software allows for peak picking, feature detection and metabolite assignment against the KEGG pathway database. Assignments were further confirmed against chemical formula

determination (as gleaned from isotopic patterns and accurate intact mass), and retention times against a 619 standard compounds library including nucleosides and nucleotide phosphates (SIGMA Aldrich, St. Louis, MO, USA; IROATech, Bolton, MA, USA)⁹³.

Relative quantitation was performed by exporting integrated peak areas values into GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA) for statistical analysis (One way ANOVA with Tukey multiple column comparison test; significance threshold for *p-values* < 0.01).

2.2.3 Mouse Subjects

Eight to ten-week-old C57BL/6 wild-type (WT) mice were purchased from Harlan Laboratories (Indianapolis, IN). Ecto-5'-nucleotidase (CD73)-deficient mice and A2B adenosine receptor (ADORA2B)-deficient mice with C57BL/6 background were generated and genotyped as described before ⁹⁴. A novel line of mice with erythrocyte specific deletion of *Adora2b* was generated by crossing mice homozygous for a floxed *Adora2b* allele with mice expressing Cre recombinase under the control of erythropoietin receptor (EpoR) gene regulatory elements⁹⁵. All protocols involving animal studies were reviewed and approved by the Institutional Animal Welfare Committee of the University of Texas Health Science Center at Houston.

2.2.4 Blood collection and preparation

Human and mouse blood, respectively, were collected and stored as described before ^{96,97}. Approximately 4 ml human blood was collected with heparin

as an anti-coagulant for 2,3-bisphosphoglycerate (2,3-BPG) measurement (Roche) as previous ⁹⁶. A portion of human blood was collected with EDTA as an anti-coagulant for complete blood count (CBC). For plasma adenosine assay one ml of the EDTA collected blood was aliquoted to tubes containing 10 μ M dipyridamole (an inhibitor of equilibrative nucleoside transporters) and 10 μ M deoxycoformycin (an inhibitor of adenosine deaminase). Approximately one ml Mouse blood was collected similar to human blood as described above⁹⁸. Human blood was frozen at -80°C after collection and during shipment from the field until assay.

2.2.5 Measurement of soluble CD73 activity in mouse

sCD73 enzyme activity was measured by quantifying the rate of ethenoadenosine (E-ADO) as described previously⁹⁴. In brief, isolated plasma were used for sCD73-specific activity measurement. First, 20 μ l plasma was preincubated at room temperature with 200 nM deoxycoformycin (an inhibitor of AMP-deaminase) in 0.1 M HEPES (pH 7.4), with 50 μ M MgCl, with or without α,β -methylene ADP (APCP, Sigma-Aldrich, a specific inhibitor of CD73). Then, samples were incubated at 37°C for 30 min in the presence of 100 μ M E-AMP. sCD73 activity was measured by determining E-ADO concentrations with reversed phase HPLC as describe before. Absorbance was measured at 260 nm. sCD73 activity was expressed as the rate of E-ADO produced from E-AMP that is inhibited by APCP.

2.2.6 Measurement of soluble CD73 activity in human

Circulating CD73 enzyme activity in human was measured by quantifying the rate of conversion of [^3H]-AMP to [^3H]-adenosine as described previously^{99, 100}. Briefly, sCD73 activity was measured after incubation of plasma with 300 μM [^3H]-AMP (Quotient BioResearch; GE Healthcare, Rushden, UK) for 60 min at 37°C. Mixture then was applied onto Alugram SIL G/UV254 sheets (Macherey-Nagel, Germany). Radiolabelled substrates and their products were separated by thin layer chromatography (TLC) and quantified by scintillation β -counting.

2.2.7 Plasma adenosine measurement

Plasma adenosine concentration was measured by reversed phase HPLC as previously described^{96, 98}. 500 μl plasma was used to isolate adenosine by sequentially adding 0.6 M cold perchloric acid, 3 M KHCO_3 /3.6 N KOH, 1.8 M ammonium dihydrogen phosphate (pH 5.1) and phosphoric acid (30%). Next, the sample was centrifuged at 20,000 $\times g$ for 5 min and the supernatant was transferred to a new tube and ready for reversed phase HPLC analysis. Adenosine content was normalized to volume and expressed as a concentration.

2.2.8 Isolation of total erythrocytes and treatment of mouse erythrocytes *in vitro*

Blood was collected with heparin as an anti-coagulant, and RBCs were isolated by centrifugation at 240 $\times g$ for 10 min at room temperature. Packed red blood cells (RBCs) were washed 3 times with culture media (F-10 nutrients mix, Life Technology) and re-suspended to 4% hematocrit (HCT). One ml of RBCs was added to each well of a 12-well plate⁹⁶. Mouse RBCs were treated with AICAR

(TOCRIS, USA) or AICAR (Sigma, USA) at 1mM for 4h under normoxic conditions¹⁰¹. At the end of experiments, 2,3-BPG levels were measured, the hemoglobin-O₂ binding affinity were analyzed and calculated as P50 by Hemox analyzer.

2.2.9 Hypoxyprobe immunohistochemistry in multiple organs with quantification

Tissue hypoxia levels were assessed by Hypoxyprobe immunohistochemistry as described before^{102, 103}. Briefly, animals were administered Hypoxyprobe (Hypoxyprobe, Inc.) via intraperitoneal injection (60 mg/kg body weight). One hour after injection, tissues including hearts, lungs and kidneys were harvested, fixed overnight in 4% buffered formalin, and embedded in paraffin. According to the manufacturer's instructions (Hypoxyprobe-1 Omni-Kit), sections of 4µm were cut and mounted on glass slides, deparaffinized through serial baths in xylene and rehydrated in a graded series of alcohol and distilled water. Endogenous peroxidase activity was quenched by 10 min of incubation in a 3% hydrogen peroxide/methanol buffer. Antigen retrieval was enhanced by autoclaving slides in sodium citrate buffer (pH 6.0) at 95°C for 15 min. After blocking with a Biotin Blocking System (Dako), the slides were then incubated with rabbit anti-PAb2627AP in a humidified chamber at 4°C overnight. After the primary antibody incubation, anti-rabbit IgG ABC staining system kit (VEACTASTAIN ABS-AP, VECTOR LAB) was used according to the protocol. Slides were subsequently stained with alkaline phosphatase substrate kit (VEACTASTAIN ABS-AP, VECTOR LAB) and counterstained with hematoxylin. Quantification of the

immunohistochemical staining was performed using the Image-Pro Plus software (Media Cybernetics, Bethesda, MD). The density of the red staining was measured. The average densities of 20 areas per samples were determined and the SEM is indicated. (n=6)

2.2.10 Immunoprecipitation

Erythrocyte pellets were lysed in lysis buffer (1X TBS, 1% NP-40, 5 mM EDTA), protease inhibitor cocktail (Sigma-Aldrich, MO), and phosphatase inhibitor cocktail (Sigma-Aldrich, MO). The total protein concentration was measured with a Protein Assay Kit (Bio-Rad). Cell lysates were precleared with 50 μ l Protein A/G Sepharose High Performance beads (GE Healthcare Life Sciences), and then incubated overnight at 4°C with 50 μ l antibody-bound beads prepared according to manufacturer's instructions and rocked gently. After immunoprecipitation, the beads were washed 4 times with 1XTBS, and boiled with 2X Laemmli buffer for Western blot analysis¹⁰⁴.

2.2.11 Western blot analysis

Proteins in human and mice erythrocytes were analyzed by western blotting. Frozen erythrocyte pellets were used for protein extraction following the protocol as mentioned above. Proteins were run on 5-20% SDS-PAGE gels and transferred to nitrocellulose membrane. After blocking (Odyssey Blocking Buffer, LI-COR), membranes were incubated with antibody against BPG mutase (Santa Cruz), or ADORA2B (Santa Cruz), or *p*-AMPK substrate (Cell Signaling), or AMPK α and *p*-AMPK α (Cell Signaling) respectively as primary antibody. Afterwards, the

membranes were probed with secondary antibodies antibody labeled with IRDye fluorophores (LI-COR), subsequently scanned and detected by using the ODYSSEY imaging system and software (LI-COR)⁹⁷.

2.2.12 ELISA measurement of erythrocyte phosphorylation of AMPK α at Thr172

Erythrocyte protein extraction from human blood were collected as described above. Next, phosphorylation levels of erythrocyte AMPK α at Thr172 were quantitatively measured by using commercially available ELISA kits. (A Solid phase sandwich ELISA, Cell Signaling)

2.2.13 Mouse bone marrow cell culture to induce erythroid progenitor cells and flow cytometry

Bone marrow cells (BMCs) were collected by flushing femur and tibias with PBS from *EpoR-Cre⁺*, *Adora2b^{fl/fl}/EpoR-Cre⁺*, and *Adora2b^{-/-}* mice (8 to 10-week-old), and single-cell suspensions were obtained by passing through a cell strainer and 21-gauge needle¹⁰⁵. Next, hematopoietic progenitor cells (HPCs) were enriched by negative selection using a mouse hematopoietic progenitor cell enrichment kit (Easy Sep, StemCell Technologies)¹⁰⁶. Briefly, isolated BMCs were first incubated with a series of biotin-conjugated lineage antibodies (Abs) including CD5, CD11b, CD19, CD45R, Ly-6G/C, and TER119 Abs, subsequently incubated with magnetic beads. The differentiated cells expressing those lineage markers bounded with magnetic beads were separated from undifferentiated HPCs by magnetic bar. Then enriched HPCs lacking those lineage markers were further

isolated by centrifugation. Purified HPCs were further seeded in wells (1x10⁵ cells/ml), and cultured as previously described with modification for erythroid cells differentiation¹⁰⁶. In brief, cells were cultured in StemSpan™ Serum-Free Expansion Medium (BSA, recombinant human insulin, human Transferrin, 2-Mercaptoethanol, Iscove's MDM) mixed with 15% FBS, 50 units/ml penicillin, 50ug/ml streptomycin, soluble erythropoietic factors and 2 units/ml EPO at 37°C in a humidified atmosphere. After 2 days, the attached differentiated cells were harvested. The percentage of early stage of erythroid progenitor cells were determined by flow cytometry using FITC-conjugated CD71 (erythroid precursors marker)^{106, 107}. Harvested erythroid progenitor cells were used for quantification of *adora2b* mRNA level by qRT-PCR as below.

2.2.14 qRT-PCR analysis of *Adora2b* mRNA in harvested erythroid progenitor cells and tissues

RNA were extracted from harvested erythroid progenitor cells and mouse lungs, then reverse transcription and real time-PCR were performed as previously described^{97, 108}. SYBR green was used for analysis of all the genes, and the relative RNA levels were quantified as the $\Delta\Delta CT$ method¹⁰⁹. Primers: Mouse *Adora2b*: forward; 5'-GCGAGAGGGATCATTGCTG-3' and reverse; 5'-CAGGAACGGAGTCAATCCAA-3', Mouse *Gapdh*: forward; 5'-TGACCTCAACTACATGGTCTACA-3' and reverse; 5'-CTTCCCATTCTCGGCCTTG-3'.

2.2.15 Statistics

All data are expressed as the mean \pm SEM. Data were analyzed for statistical significance using GraphPad Prism 5 software (GraphPad Software). Two-tailed Student's *t* tests (paired or unpaired as appropriate) were applied in 2-group analysis. Differences between the means of multiple groups were compared by one-way analysis of variance, followed by a Turkey's multiple comparisons test. P value of less than 0.05 was considered significant. The relationship between two variables X and Y were analyzed by Pearson product-moment correlation coefficient method. $P < 0.05$ (two-sided) was considered statistically significant. The linear correlation (dependence) is described as R square.

2.3 Results

2.3.1 Metabolomic screening reveals that altitude hypoxia activates the Rapoport-Luebering Shunt in erythrocytes resulting in increased production of 2,3-BPG and increased oxygen release from hemoglobin

To identify metabolic alterations in erythrocyte in response to hypoxia, we conducted nonbiased high throughput metabolomic profiling of erythrocytes from humans at sea level and at high altitude. Specifically, we recruited 21 young healthy lowland volunteers who were examined at sea level and within two hours of arrival at an altitude of approximately 5260 meters (ALT1) and on day 16 at high altitude (ALT16) (Fig. 3). Overall, 233 metabolites were confidently identified and relatively quantified out of over 9000 detected features (mass to charge ratios) in the erythrocytes under sea level conditions and at high altitude (Table 2). Analysis of these metabolic changes revealed that the erythrocyte-specific Rapoport-Luebering shunt, occurring at a branch point in the pathway of anaerobic glycolysis for production of 2,3-BPG from 1,3-bisphoglycerate (1,3-BPG) (Fig. 4A), was rapidly activated by high-altitude hypoxia. Specifically, levels of 2,3-BPG and its upstream glycolytic intermediate, glyceraldehyde-3-phosphate (G3P), were increased within 2 hr of arrival at high altitude (ALT1) and were further elevated

on day 16 (ALT16). In contrast, the levels of the erythrocyte monophosphoglycerates (3-PG and 2-PG) and phosphoenolpyruvate (PEP), the three immediate downstream products of 2,3-BPG, were significantly reduced on day 1 and further reduced on day 16 at high altitude compared to sea level (Fig. 4B-E). To further validate our metabolomic screening, we accurately quantified erythrocyte 2,3-BPG levels in human samples. Consistently, we found that erythrocyte 2,3-BPG levels were significantly induced within two hours at high altitude hypoxia and further elevated on day 16 at high altitude (Fig. 5). Thus, our results indicate that in response to high altitude hypoxia the Rapoport-Luebering Shunt is activated resulting in a significant portion of G3P being utilized to produce 2,3-BPG rather than directly metabolized to the glycolytic intermediate, 3-PG (Fig. 5).

Because 2,3-BPG is an allosteric modulator that decreases Hb-O₂ binding affinity and thereby promotes O₂ release⁸⁷, it is possible that hypoxia-induced 2,3-BPG production in erythrocytes triggers O₂ release. To test this possibility, we quantified the O₂ release capacity of erythrocytes by accurately measuring P50, the partial pressure of O₂ required to achieve 50% Hb-O₂ saturation, from human subjects at high altitude. We found that erythrocyte P50 values were significantly elevated in human subjects after prolonged residence at that altitude (ALT16) compared to two hours of arrival at 5260 m (ALT1). Finally, the ratio of

(ALT16/ALT1) in erythrocyte 2,3-BPG levels upon extended stay at 5260 m was significantly correlated with the ratio of (ALT16/ALT1) in erythrocyte P50, reflecting reduced Hb-O₂ binding affinity and enhanced erythrocyte O₂ release capacity. No differences were noted between male and female subjects (Fig. 6). These findings provide evidence that high altitude hypoxia coordinately induces erythrocyte 2,3-BPG production and P50 in humans adapting to high altitude (Fig. 4A).

	Male	Female
Number	12	9
Age(Years)	21.08±1.24	20.44±1.51
HT(cm)	181.30±4.06	168.48±5.18
WT(kg)	75.29±6.26	62.31±6.43
BMI(kg/m ²)	22.85±1.67	21.88±1.84

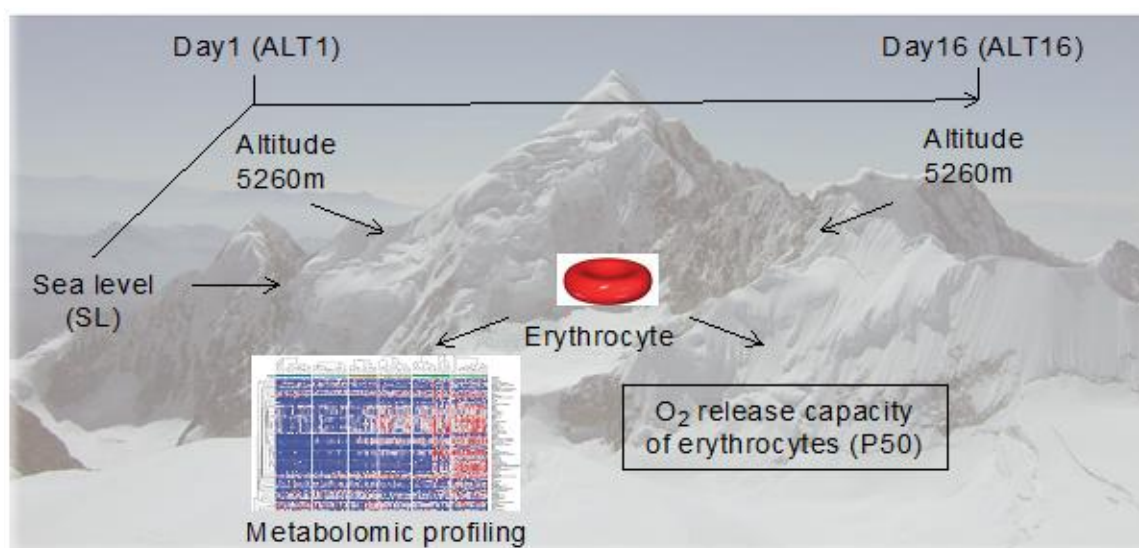


Figure.3. Table of human volunteer characteristics including age, height (HT), weight (WT), body mass index (BMI), and schema for high altitude human studies. (n=21).

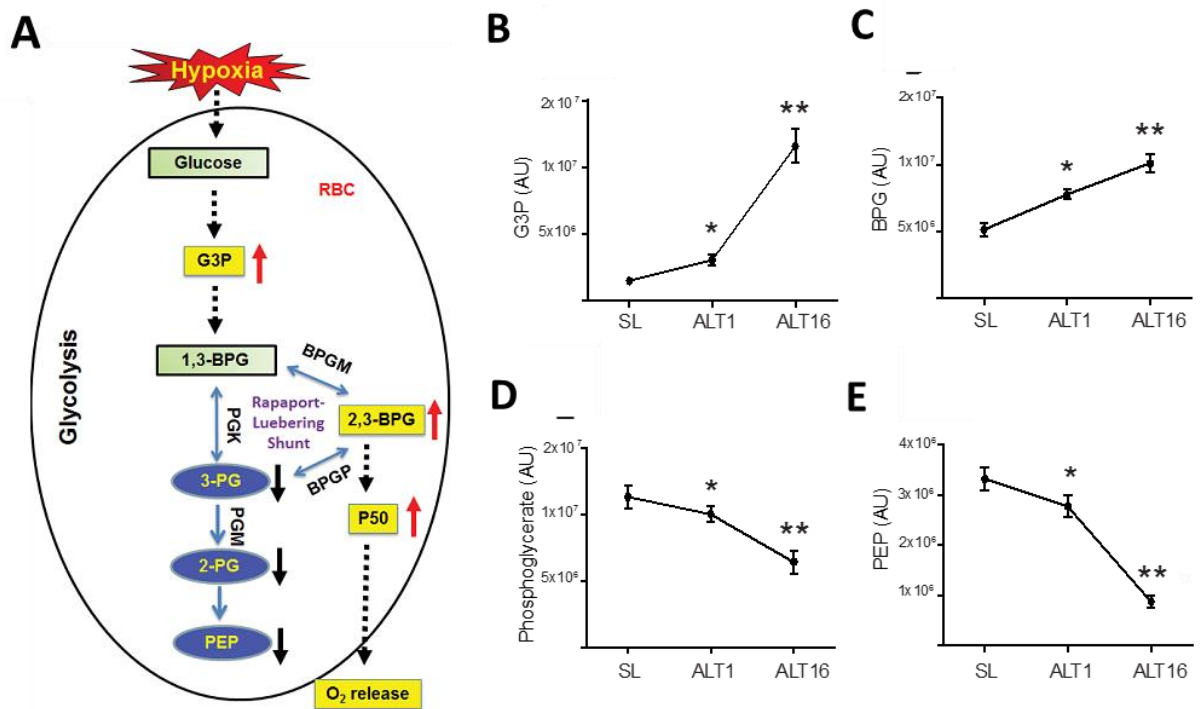


Figure.4. (A) Schematic drawing of erythrocyte-specific Rapoport-Luebering shunt occurring at a branch point in the pathway of anaerobic glycolysis for 2,3-bisphosphoglycerate (2,3-BPG) production. (B-E) Metabolomic profiling reveals the significant changes in the relative erythrocyte concentration of glyceraldehyde-3-phosphate (G3P) (B), bisphosphoglycerate (BPG) (C), the monophosphoglycerates, 2-phosphoglycerate (2-PG) and 3-phosphoglycerate (3-PG) (D) and phosphoenopyruvate (PEP) (E) at sea level (SL) and at high altitude on day 1 and day 16. Data are expressed as mean \pm SEM; * P <0.05 vs SL; ** P <0.05 vs ALT1. AU (area under the peak)

2.3.2 Extracellular adenosine concentrations and soluble CD73 activity increase in humans at high altitude and increased adenosine is correlated with elevated erythrocyte 2,3-BPG and O₂ release capacity

Specific factors and signaling pathways involved in 2,3-BPG induction and subsequent O₂ release from erythrocytes under high altitude has not been previously identified. Recent studies show that increased circulating adenosine is involved in 2,3-BPG induction in erythrocytes of sickle cell disease (SCD)⁹⁶. However, whether adenosine signaling contributes to erythrocyte 2,3-BPG induction and subsequent O₂ release underlying high altitude hypoxia adaptation remains unknown. To test this hypothesis, we used high performance liquid chromatography (HPLC) to measure plasma adenosine levels in the 21 lowland volunteers at sea level and following ascent to high altitude. We found that the circulating adenosine levels were elevated within two hours of arrival at high altitude compared to sea level and were further elevated on ALT16 following an extended stay at 5260 m (Fig. 5). No significant differences in elevated plasma adenosine levels were observed between males and females (Fig. 6). Ecto-5'-nucleotidase (CD73) is an ectoenzyme anchored to the cell surface that plays a key role in the synthesis of extracellular adenosine from AMP⁷⁵. Under certain circumstances, the ectoenzyme can be cleaved from the cell surface and exist in the circulation as a soluble nucleotidase¹¹⁰. Therefore, we further measured soluble CD73 (sCD73) activity in normal individuals at sea level and at high altitude. Compared to sea level, sCD73 activity was significantly increased in the human subjects within two hours following arrival at 5260 m (ALT1) and further increased

following an extended stay at high altitude (AT16) (Fig. 5). There was no significant difference in elevated sCD73 activity between males and females in response to high altitude (Fig. 6).

Taken together, these results show that plasma adenosine concentration and sCD73 activity increases rapidly in response to high-altitude hypoxia and that the induction of plasma adenosine correlates with the induction of erythrocyte 2,3-BPG level and O₂ releasing capacity.

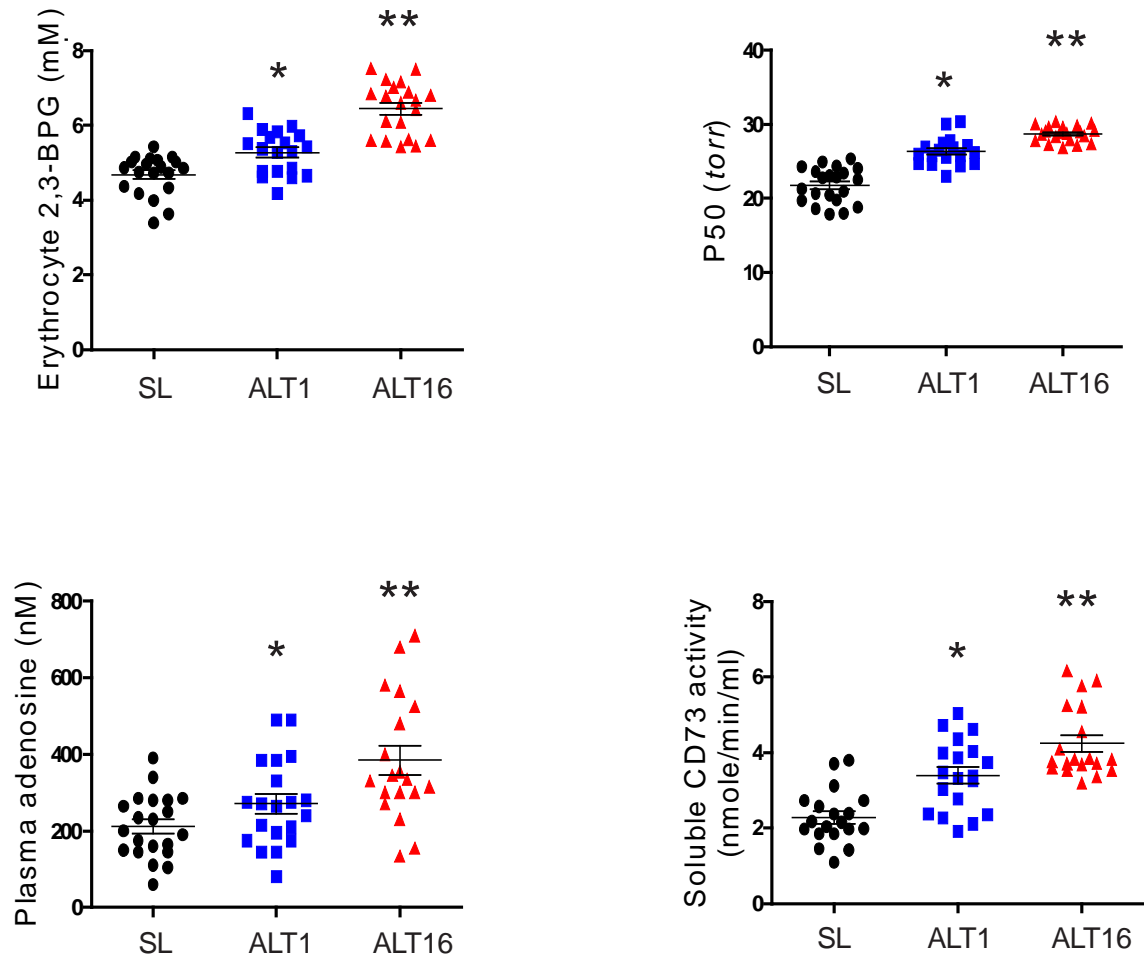


Figure. 5. Erythrocyte 2,3-BPG concentration, P50 level, plasma adenosine concentration and soluble CD73 activity (sCD73) were elevated at high altitude hypoxia on ALT1 and ALT16 over SL. Data are expressed as mean \pm SEM; * $P < 0.05$ vs SL; ** $P < 0.05$ vs ALT1.

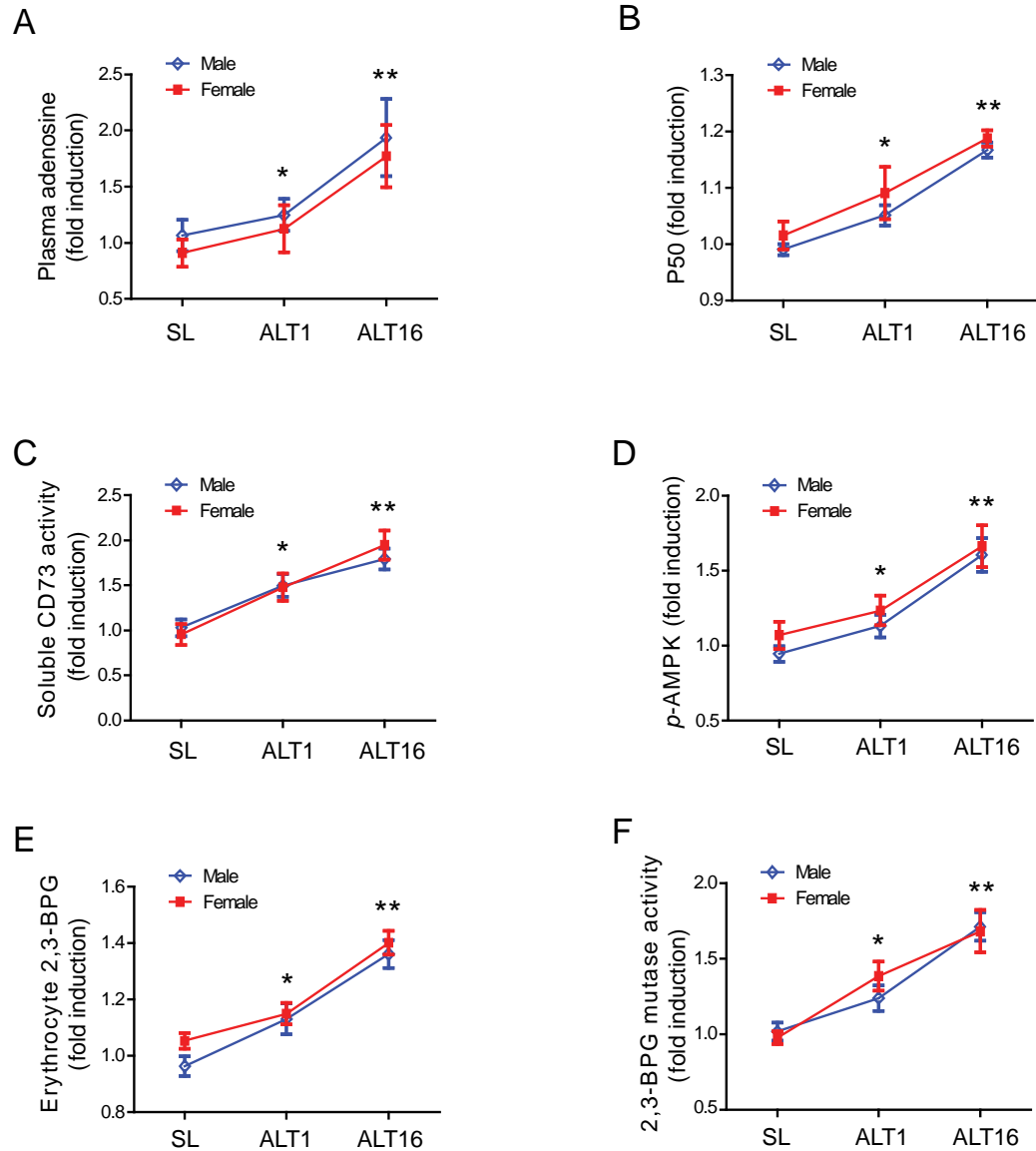


Figure. 6. No significant differences are observed between male and female at high altitude hypoxia in elevated erythrocyte 2,3-BPG concentration, elevated P50 levels, elevated plasma adenosine concentration, elevated soluble CD73 activity, elevated *p*-AMPK levels and elevated 2,3-BPG mutase

activity. (A-F) Erythrocyte 2,3-BPG concentration, P50 level, plasma adenosine concentration, soluble CD73 activity, p-AMPK levels, and 2,3-BPG mutase activity were significantly elevated both in male and female at high altitude hypoxia, and there were no significant differences between male and female. Data are expressed as mean \pm SEM; *P<0.05 vs SL; **P<0.05 vs ALT1.

2.3.3. Soluble CD73 activity is induced under hypoxia and elevated CD73 is essential for hypoxia-induced production of plasma adenosine and increased erythrocyte 2,3-BPG level and O₂ releasing capacity

Although extracellular adenosine is well-known to orchestrate a physiological response to tissue injury and hypoxia, nothing is known about the functional role of plasma adenosine in the normal erythrocyte under hypoxia. Our human studies presented above raise an intriguing hypothesis that elevated plasma adenosine is dependent on elevated CD73 and that elevated adenosine has a previously unrecognized role in high altitude adaption by regulating 2,3-BPG production and Hb-O₂ binding affinity in erythrocytes. To test this hypothesis, we exposed WT and CD73-deficient mice (*Cd73*^{-/-}) to hypoxia (10% oxygen, similar to that at 5260 m) for one week. Similar to the human high-altitude studies, we found that sCD73 activity, plasma adenosine levels, erythrocyte 2,3-BPG and P50 were significantly increased in WT mice following 1 week of hypoxia compared to normoxia (Fig. 7A-D). In contrast, hypoxia-mediated increased plasma adenosine, erythrocyte 2,3-BPG and erythrocyte O₂ releasing capacity were significantly impaired in *Cd73*^{-/-} mice (Fig. 7A-D). These results indicate that in mice sCD73 activity is induced by hypoxia, as seen in human volunteers at high altitude, and that sCD73 is required for hypoxia-induced plasma adenosine production and elevated erythrocyte 2,3-BPG levels and O₂ releasing capacity.

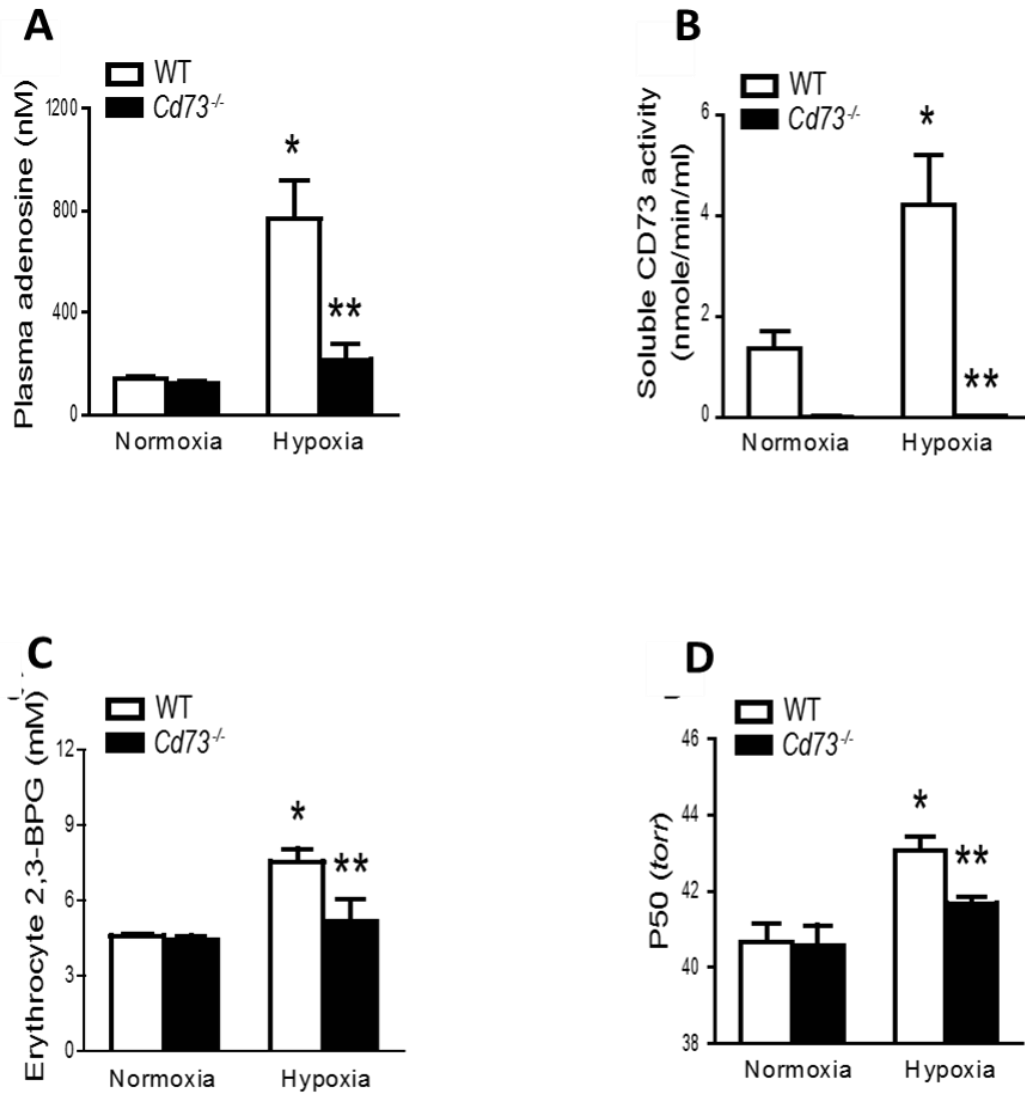


Figure. 7 CD73 is essential for hypoxia-induced plasma adenosine, erythrocyte 2,3-BPG concentration and P50 levels in mice. Plasma adenosine concentration (**A**), soluble CD73 activity (**B**), Erythrocyte 2,3-BPG (**C**), and P50 (**D**) in WT mice and *Cd73*^{-/-} mice under normoxia or hypoxia (10% O₂ 1 week). Data are expressed as mean \pm SEM; **P*<0.05 vs WT under normoxia; ***P*<0.05 vs WT under hypoxia (n=10).

2.3.4 CD73-mediated elevated extracellular adenosine protects against tissue hypoxia in mice

To assess the functional importance of CD73-mediated increased plasma adenosine in tissue, we measured the tissue hypoxia levels using a hypoxyprobe in WT mice and *Cd73*^{-/-} mice under normoxic conditions and following one week of hypoxia as described above. No hypoxia signals were seen in tissue sections of *Cd73*^{-/-} or WT mice under normoxic conditions (Fig. 8A). However, under hypoxic conditions, immunohistochemical (IHC) analysis with the hypoxyprobe revealed that multiple organs including kidneys, lungs and hearts displayed mild hypoxic signal in WT mice (Fig. 8A). In contrast, 7 days at 10% O₂ led to severe hypoxia in various organs including kidneys, lungs and hearts of *Cd73*^{-/-} mice (Fig. 8A). Image quantification analysis demonstrated that the intensity of hypoxyprobe in the kidneys, lungs and hearts was significantly elevated in *Cd73*^{-/-} mice compared to WT mice (Fig. 8B-D). These results show that CD73-mediated elevation of plasma adenosine plays a beneficial role to prevent tissue hypoxia in mice.

Hypoxia is known to induce lung injury including pulmonary vascular leakage and inflammation¹¹¹. Thus, we collected bronchoalveolar lavage fluid (BALF) of WT and *Cd73*^{-/-} mice for evaluation of pulmonary damage and inflammation. Specifically, we quantified cell count, albumin concentration, and interleukin 6 (IL-6) concentration in BALF of WT and *Cd73*^{-/-} mice under normoxia and hypoxia. Consistent with our tissue hypoxia staining and MPO activity measurements in the

lung, cell count, albumin and IL-6 concentrations in BALF were slightly induced by hypoxia in WT mice and their levels were significantly further increased in *Cd73*^{-/-} mice (Fig. 9A-D). Thus, these results show that CD73-mediated elevation of plasma adenosine plays a beneficial role in preventing hypoxia-induced lung damage and inflammation in mice.

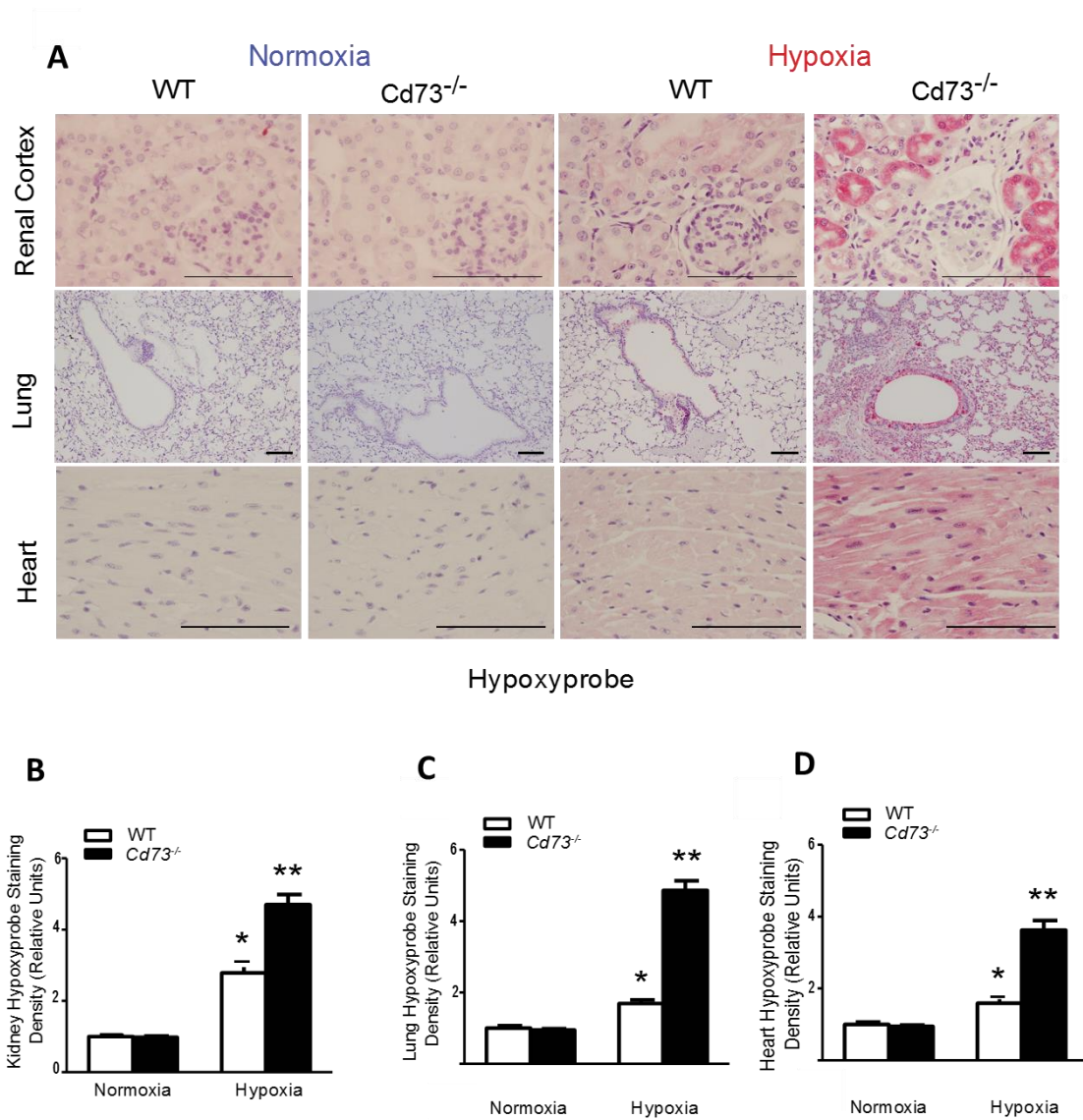


Figure. 8. (A-D) Deletion of Cd73^{-/-} results in elevated tissue hypoxia under hypoxia (10% O₂ 1 week). Immunohistochemical analysis of tissue hypoxia by hypoxyprobe. (B-D) Semi-quantification of the hypoxyprobe staining in multiple tissues including kidney, lung and heart of WT mice and Cd73^{-/-} mice under normoxia or 10% O₂ 1 week hypoxia condition. Data are expressed as mean \pm SEM; *P<0.05 vs WT under normoxia; **P<0.05 vs WT under hypoxia (n=10; Scale bar=200 μ m)

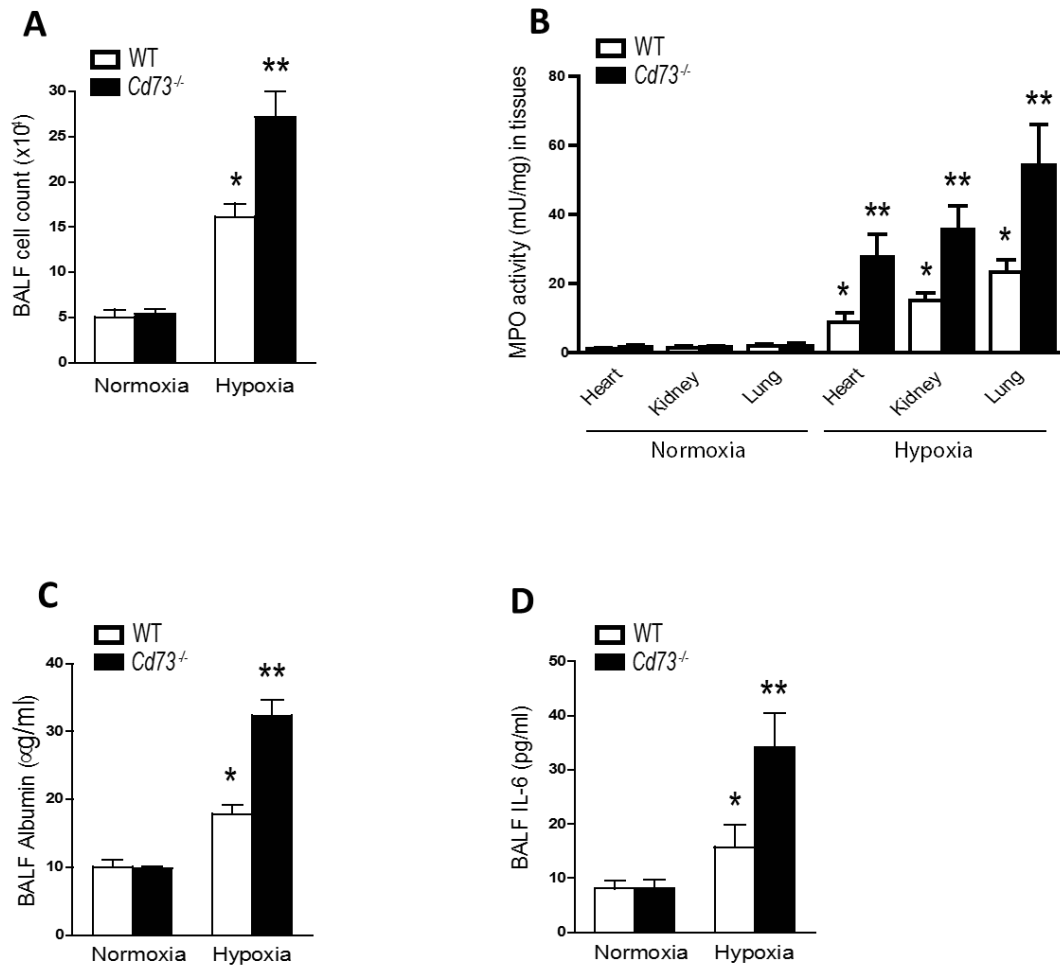


Figure 9. (A-D) Deletion of *Cd73*^{-/-} results in elevated tissue inflammation infiltration in multiple tissues, as well as pulmonary dysfunction under hypoxia (10% O₂ 1 week). Analysis of tissue damage by Myeloperoxidase activity (**B**) in kidney, lung and heart. Bronchoalveolar lavage fluid (BALF) total cell count (**A**), BALF albumin concentration (**C**), BALF interleukin 6 concentration (**D**) in WT mice and *Cd73*^{-/-} mice. Data are expressed as mean \pm SEM; **P*<0.05 vs WT under normoxia; ***P*<0.05 vs WT under hypoxia (n=10; Scale bar=200 μ m).

2.3.5 ADORA2B underlies hypoxia-induced 2,3-BPG production and oxygen release capacity in *in vitro* cultured mouse erythrocytes

It is known that increased extracellular adenosine elicits physiological or pathological functions by engaging its four adenosine receptors including ADORA1, ADORA2A, ADORA2B, and ADORA3¹¹². Each adenosine receptor has a distinct cellular or tissue distribution and affinity for adenosine¹¹³. To identify which adenosine receptor is responsible for hypoxia-induced 2,3-BPG production and O₂ release capacity in erythrocytes, we conducted genetic studies in four adenosine receptor-deficient mice. We found that hypoxia significantly induced 2,3-BPG production and O₂ release capacity in cultured erythrocytes isolated from WT mice, *Adora1*-deficient mice, *Adora2a*-deficient mice and *Adora3*-deficient mice. However, hypoxia-mediated effects were absent in cultured erythrocytes isolated from *Adora2b*-deficient mice (Fig. 11A-B), indicating that hypoxia-induced erythrocyte 2,3-BPG production and O₂ release capacity is dependent on ADORA2B.

2.3.6 Genetic deletion of erythrocyte ADORA2B attenuates hypoxia-induced 2,3-BPG production and oxygen release capacity *in vivo*

To precisely determine the importance of erythrocyte ADORA2B-mediated

elevated 2,3-BPG production and O₂ release in hypoxia, we generated mice with erythrocyte lineage-specific ablation of *Adora2b* genes by mating mice with floxed *Adora2b* alleles (*Adora2b^{ff}*) with mice containing a transgene expressing Cre recombinase only in the erythroid lineage (*EpoR-Cre⁺*). First, we validated the specificity and efficiency of ablation of *Adora2b* in erythroid cells by EpoR-Cre using bone marrow (BM) cells isolated from *Epo-Cre⁺*, *Adora2b^{ff}/EpoR-Cre⁺* and *Adora2b^{-/-}* mice. The BM-derived hematopoietic progenitor cells (HPCs) were enriched by negative selection, followed by induction to erythroid progenitor cells in erythroid-differentiation medium containing erythropoietin (Fig. 10A). After two days, we found that over 96% of harvested HPCs isolated and enriched from *Epo-Cre⁺*, *Adora2b^{ff}/EpoR-Cre⁺* and *Adora2b^{-/-}* mice were differentiated into CD71⁺ erythroid progenitor cells, which is consistent with a previous publication (Fig. 10B)¹⁰⁶. Moreover, qRT-PCR analysis showed that erythrocyte lineage *Adora2b* mRNA levels of *Adora2b^{ff}/EpoR-Cre⁺* were reduced to levels similar to that of *Adora2b^{-/-}* mice, whereas pulmonary *Adora2b* mRNA levels in *Adora2b^{ff}/EpoR-Cre⁺* were still similar to control mice (*EpoR-Cre⁺*) (Fig. 10C-D). These results demonstrated that we had successfully generated mice with erythrocyte lineage specific deletion of *Adora2b*.

To test the functional role of erythrocyte ADORA2B signaling in response to hypoxia, we exposed *EpoR-Cre* mice and *Adora2b^{ff}/EpoR-Cre⁺* mice to normoxia or hypoxia (10% O₂) for one week. The levels of plasma adenosine, erythrocyte 2,3-BPG and P50 were similar in *EpoR-Cre⁺* mice and *Adora2b^{ff}/EpoR-Cre⁺* mice under normoxia (Fig. 11C-E). Following one week of hypoxia exposure, plasma adenosine concentrations increased to similar levels in *EpoR-Cre⁺* mice and *Adora2b^{ff}/EpoR-Cre⁺* mice (Fig. 11C). However, the hypoxia-induced erythrocyte 2,3-BPG production and P50 levels observed in the *EpoR-Cre⁺* mice were significantly attenuated in *Adora2b^{ff}/EpoR-Cre⁺* mice (Fig. 11D-E). Thus, our studies provide strong genetic evidence that mouse erythrocyte ADORA2B is essential for elevated adenosine-mediated induction of erythrocyte 2,3-BPG and O₂ releasing capacity under hypoxia.

2.3.7 Erythrocyte ADORA2B is essential to protect against tissue hypoxia, inflammation and lung damage

To further determine if erythrocyte ADORA2B signaling is tissue protective under hypoxia, we assessed tissue hypoxia by hypoxyprobe in *EpoR-Cre⁺* mice and *Adora2b^{ff}/EpoR-Cre⁺* mice following one week of hypoxia (10% O₂). We found that hypoxia treatment led to severe tissue hypoxia in various organs including

kidneys, lungs and hearts in *Adora2b^{ff}/EpoR-Cre⁺* mice, while only a mild hypoxia signal was present in those tissues of *EpoR-Cre⁺* mice (Fig. 12A). Image quantification analysis demonstrated that the intensity of hypoxyprobe signals in kidneys, lungs and hearts were significantly elevated in *Adora2b^{ff}/EpoR-Cre⁺* mice compared to *EpoR-Cre⁺* (Fig. 12B-D) following hypoxia challenge. We also observed greater elevation of MPO activity in kidney, lung and heart of *Adora2b^{ff}/EpoR-Cre⁺* mice compared to *EpoR-Cre⁺* mice after hypoxia exposure (Fig. 13A). Furthermore, greater lung injury reflected by increased cell counts, albumin and IL-6 concentrations in BALF were detected in *Adora2b^{ff}/EpoR-Cre⁺* mice after hypoxia challenge (Fig. 13B-D). These findings demonstrate that erythrocyte ADORA2B is essential for tissue protection during hypoxia exposure.

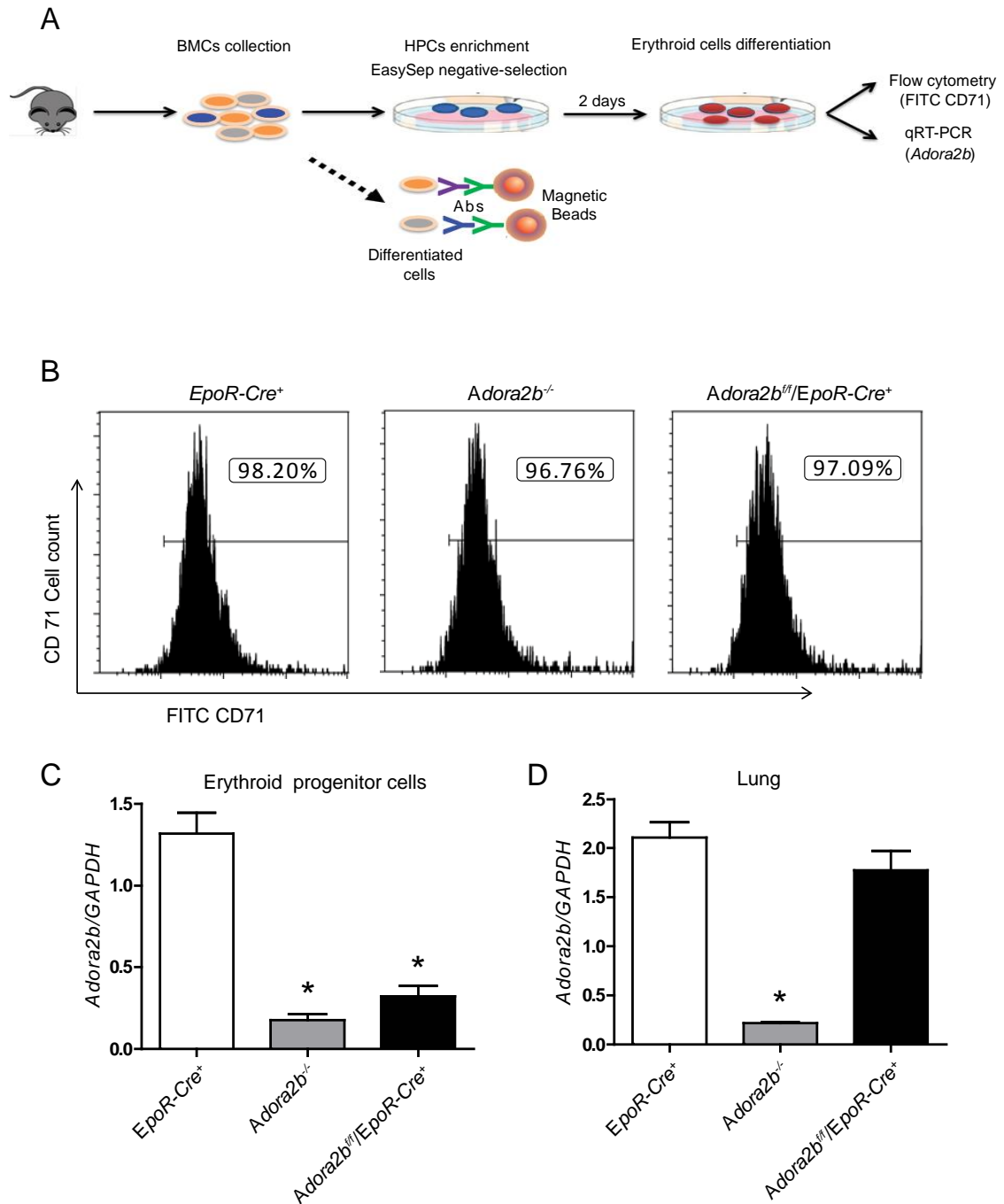


Figure. 10. Effective and specific deletion of *Adora2b* gene in erythrocyte lineage. (A) Schema of generation of erythrocyte specific deletion of *Adora2b* mice by cre/loxP system. (B) Schema of mouse bone marrow-derived erythropoietic culture. (C) The percentage of harvested erythroid cells labeled with FITC-

conjugated CD71 in erythroid progenitor cells of *EpoR-Cre*⁺, *Adora2b*^{-/-} and *Adora2b*^{fl/fl}/*EpoR-Cre*⁺ mice. (D-E) Relative *Adora2b* mRNA levels in erythroid progenitor cells and lung of *EpoR-Cre*⁺ mice, *Adora2b*^{-/-} and *Adora2b*^{fl/fl}/*EpoR-Cre*⁺ mice. Data are expressed as mean \pm SEM; *P<0.05 vs *EpoR-Cre*⁺ mice. (n=6)

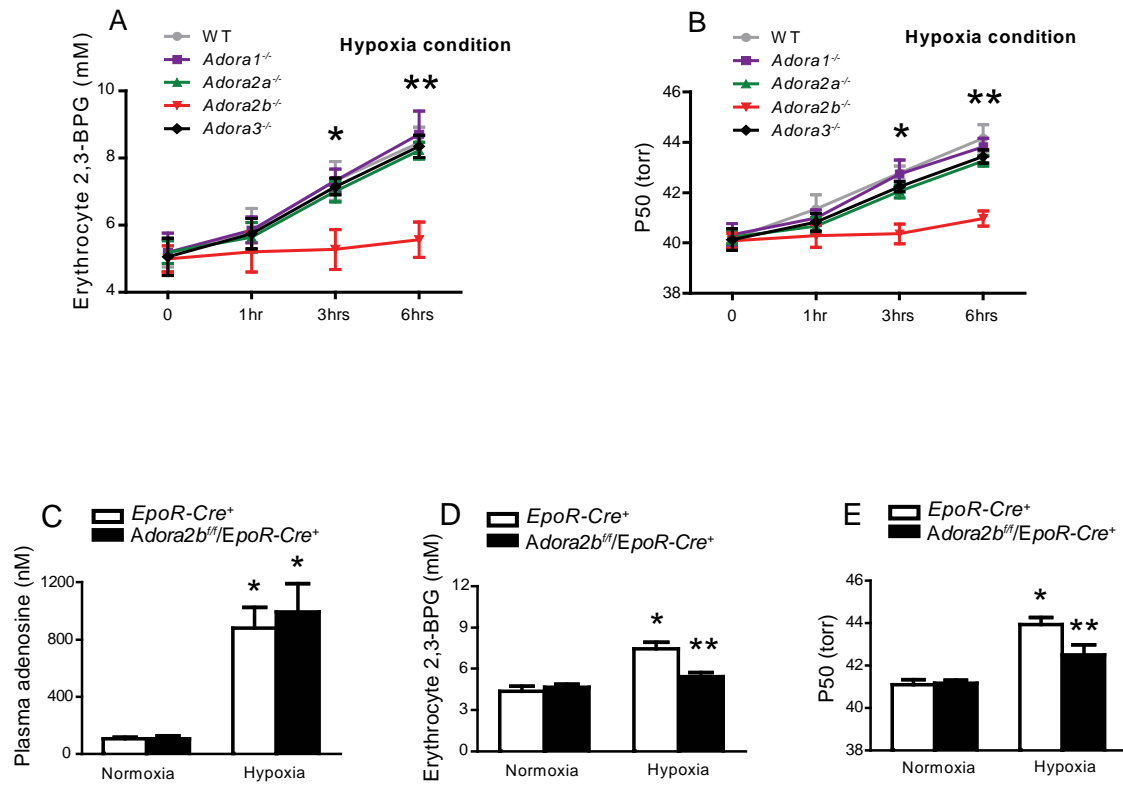


Figure. 11. (A-D) Erythrocyte ADORA2B activation induces erythrocyte 2,3-BPG production and oxygen release capacity to counteract tissue hypoxia, inflammation and lung damage in mice

(A and B) Hypoxia induces 2,3-BPG production (A) and P50 (B) levels in cultured WT, *Adora1*^{-/-}, *Adora2a*^{-/-}, *Adora3*^{-/-} but not *Adora2b*^{-/-} mouse erythrocytes in a time-dependent manner. **P*<0.05 for 3 hours hypoxia vs normoxia; ***P*<0.05 for 6 hours hypoxia vs 3 hours hypoxia (n=8). (C-E) Erythrocyte ADORA2B contributes to hypoxia-induced 2,3-BPG production and P50 levels. Plasma adenosine (C), erythrocyte 2,3-BPG (D) and P50 (E) of *EpoR-Cre*⁺ mice and *Adora2b*^{ff}/*EpoR-Cre*⁺ mice under normoxia or hypoxia (10% O₂ 1 week). Data are expressed as mean_± SEM; **P*<0.05 vs *EpoR-Cre*⁺ mice under

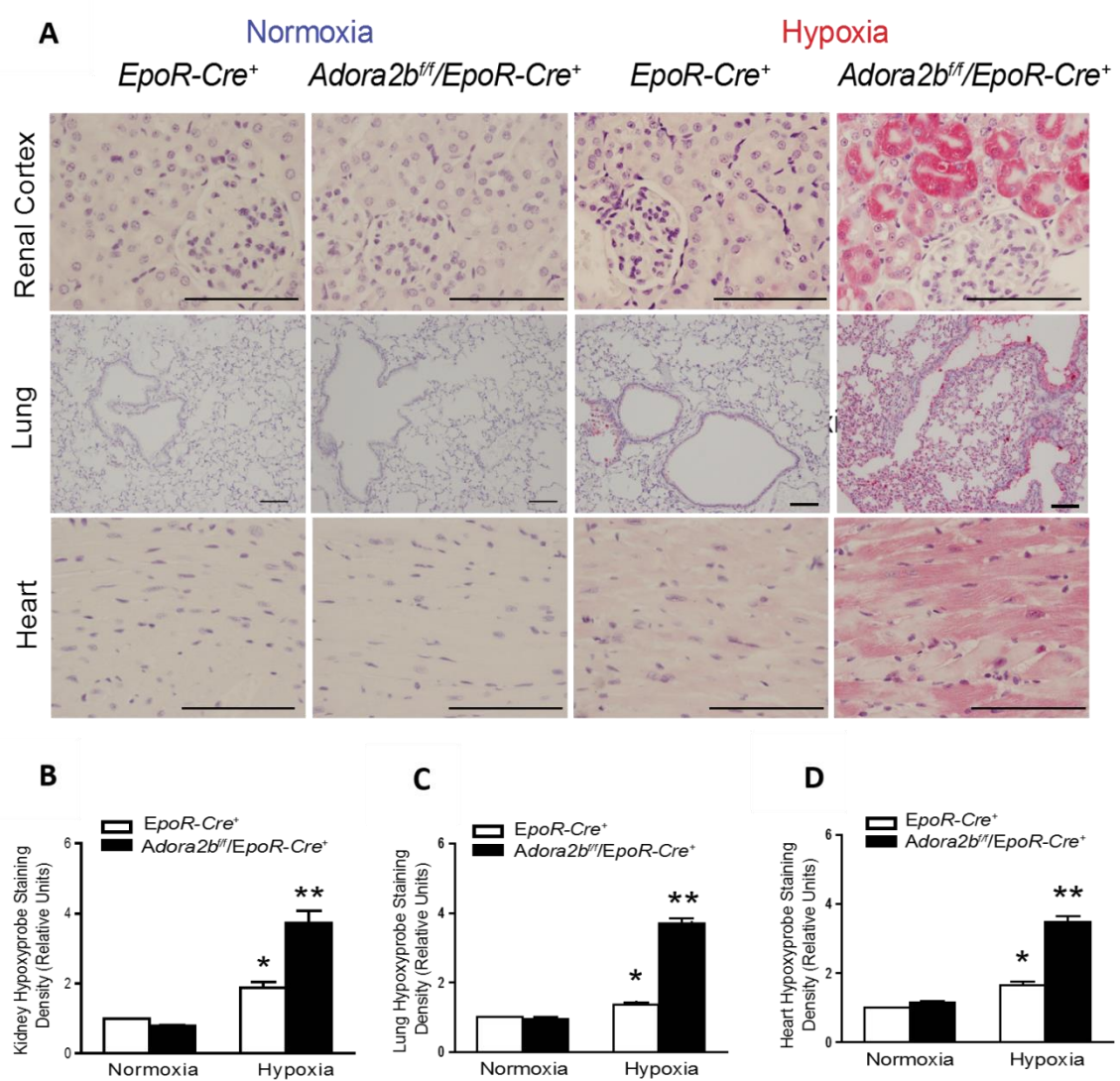


Figure. 12. Targeted deletion of erythrocyte ADORA2B results in elevated tissue hypoxia (10% O₂, 1 week). Immunohistochemical analysis of tissue hypoxia by hypoxyprobe (A). Semi-quantification of the hypoxyprobe staining in multiple tissues including kidney, lung and heart of *EpoR-Cre⁺* and *Adora2b^{fl/fl}/EpoR-Cre⁺* mice under normoxia or 10% O₂ 1 week hypoxia condition (B-D). *P<0.05 vs *EpoR-Cre⁺* mice under normoxia condition; **P<0.05 vs *EpoR-Cre⁺* mice under hypoxia condition. (n=10; Scale bar=200 μM)

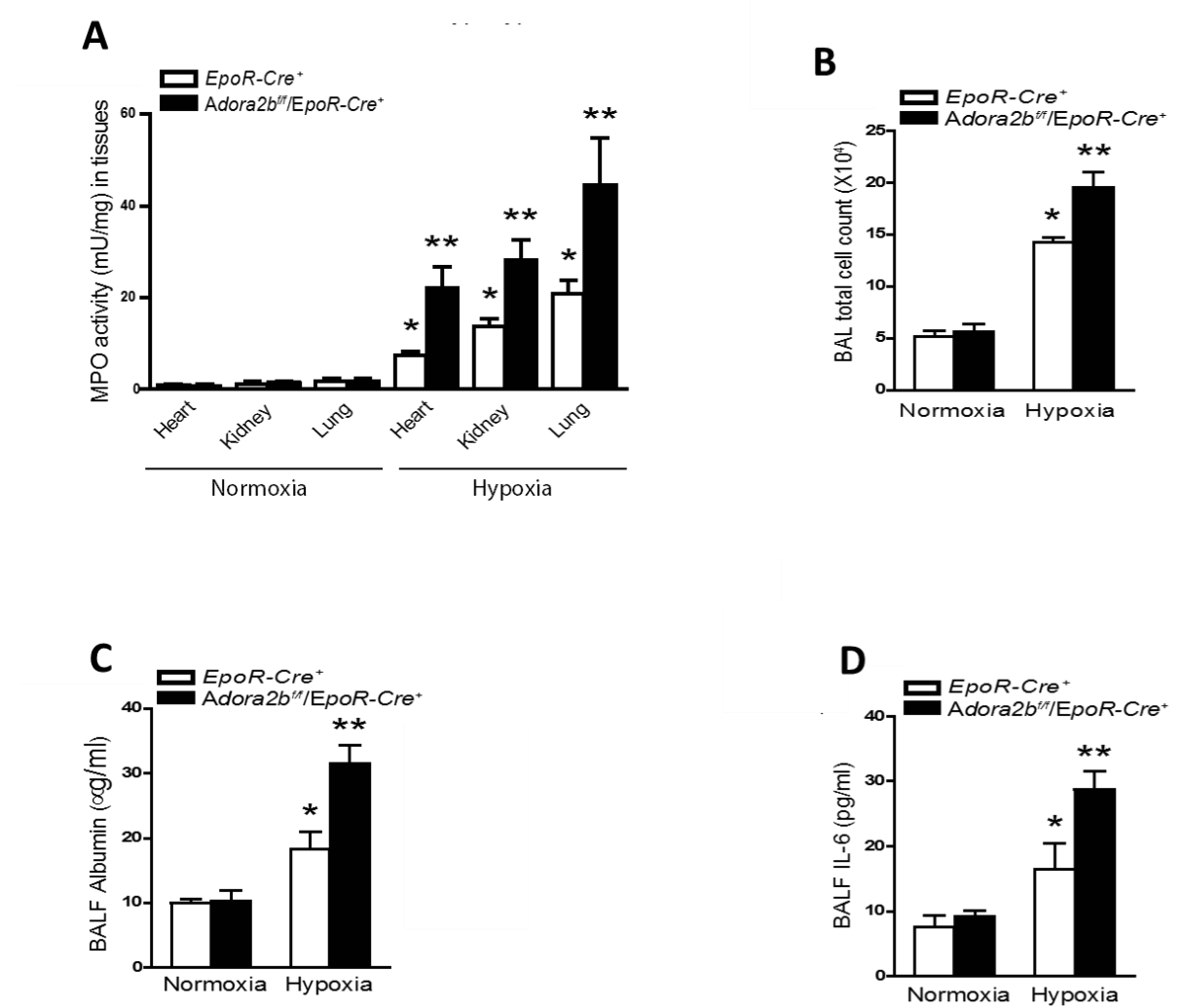


Figure. 13. Myeloperoxidase activity (A) in kidney, lung and heart. Bronchoalveolar lavage fluid (BALF) total cell count (B), BALF albumin concentration (C), BALF interleukin 6 concentration (D) in $EpoR-Cre^+$ mice and $Adora2b^{ff}/EpoR-Cre^+$ mice. Data are expressed as mean \pm SEM; * $P < 0.05$ vs $EpoR-Cre^+$ mice under normoxia; ** $P < 0.05$ vs $EpoR-Cre^+$ mice under hypoxia (n=10; Scale bar=200 μm)

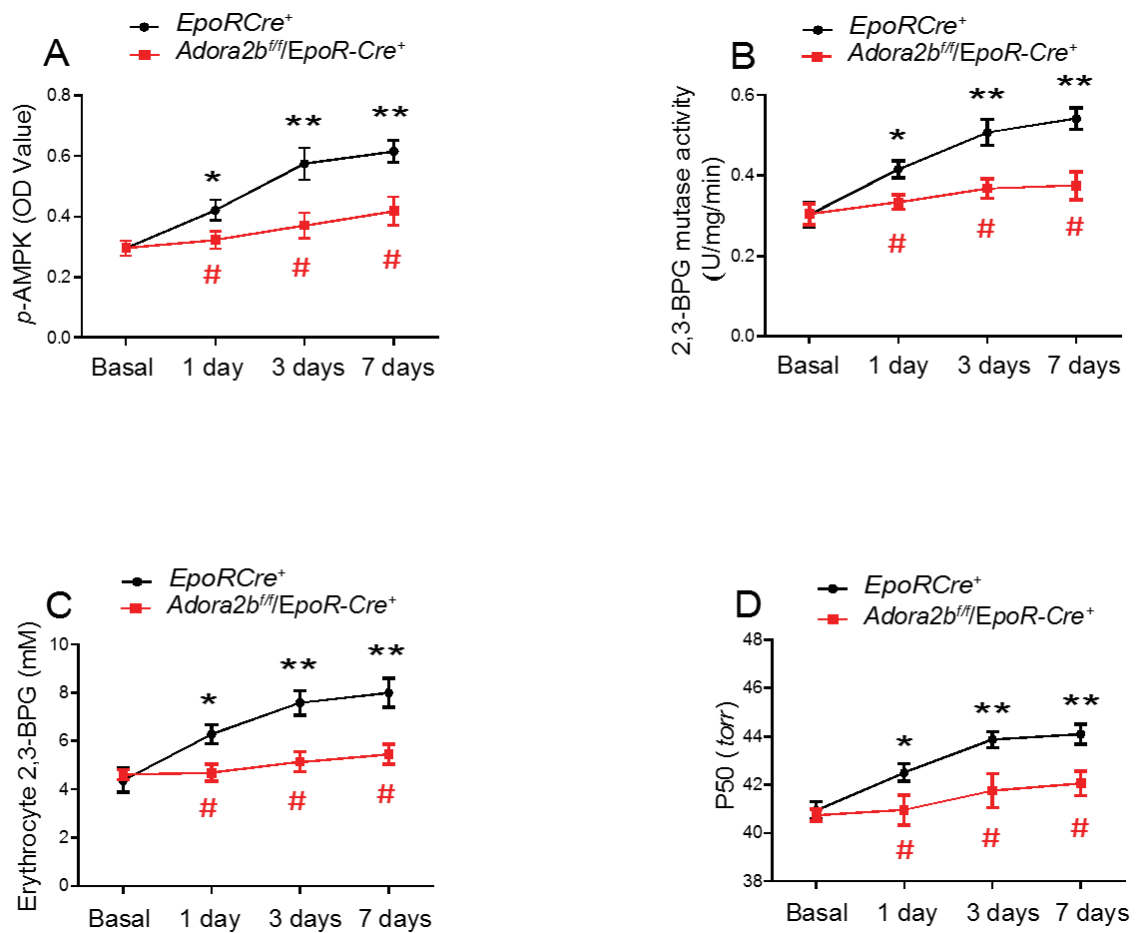


Figure 14. Erythrocyte ADORA2B is essential for hypoxia-induced *p*-AMPK, 2,3-BPG mutase activity, 2,3-BPG concentration and P50 levels *in vivo*. Erythrocyte *p*-AMPK α (quantified by ELISA) (A), 2,3-BPG mutase activity (B), 2,3-BPG concentration (C) and P50 (D) of *EpoR-Cre⁺* mice and *Adora2b^{fl/fl}/EpoR-Cre⁺* mice under normoxia or hypoxia (10% O₂, 90% N₂) for 1 day, 3 days, and 7 days. Data are expressed as mean \pm SEM; **P*<0.05 vs *EpoR-Cre⁺* mice under normoxia; ***P*<0.05 vs *EpoR-Cre⁺* mice under hypoxia for 1 day; #*P*<0.05 *Adora2b^{fl/fl}/EpoR-Cre⁺* mice vs *EpoR-Cre⁺* mice at the same time point (n=10).

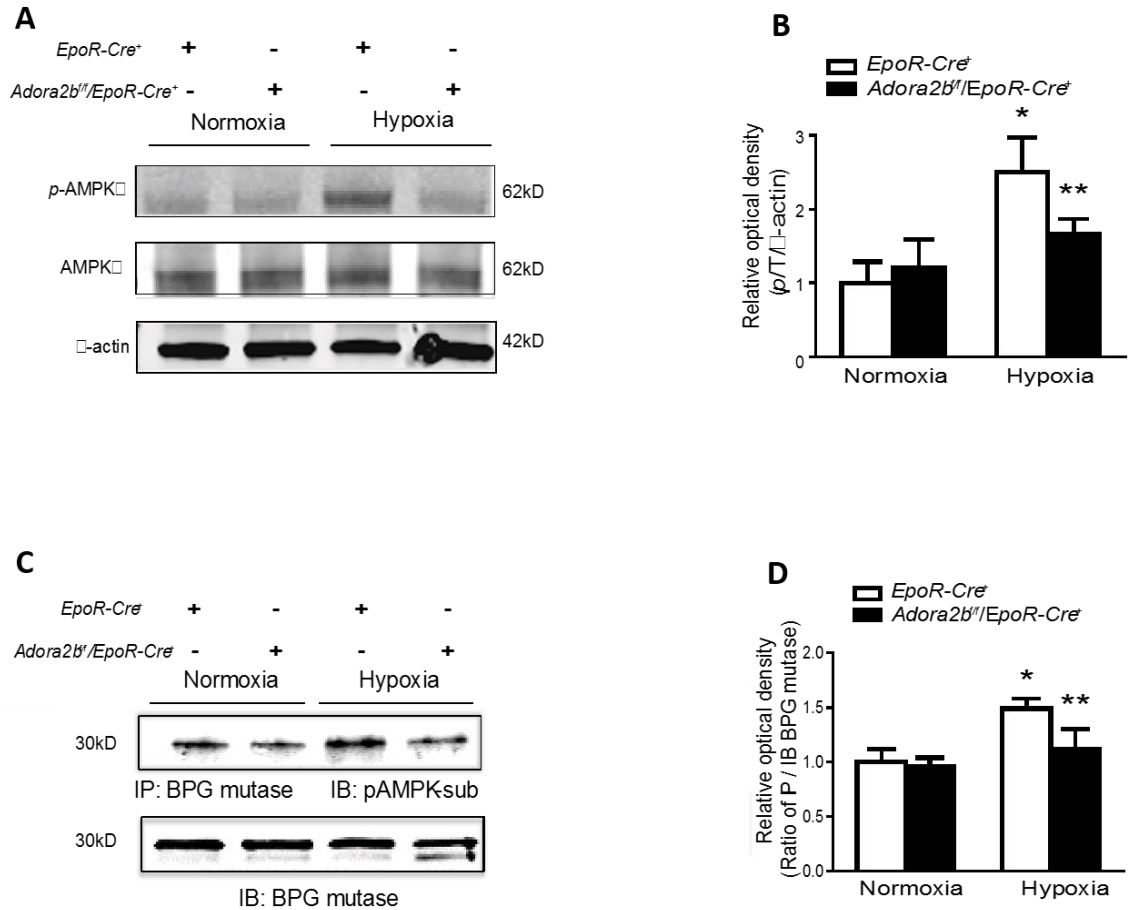


Figure 15. (E) Representative western blot and relative image quantification analysis of *p*-AMPK α in erythrocytes of *EpoR-Cre*⁺ mice and *Adora2b*^{fl/fl}/*EpoR-Cre*⁺ mice under normoxia or hypoxia (10% O₂, 90% N₂) for 1 week. (F) Representative western blot and relative image quantification analysis of *p*-AMPK phosphorylated 2,3-BPG mutase levels in the erythrocyte lysates in *EpoR-Cre*⁺ mice and *Adora2b*^{fl/fl}/*EpoR-Cre*⁺ mice under normoxia or hypoxia (n=3). Data are expressed as mean \pm SEM; **P*<0.05 vs *EpoR-Cre*⁺ mice under normoxia; ***P*<0.05 vs *EpoR-Cre*⁺ mice under hypoxia.

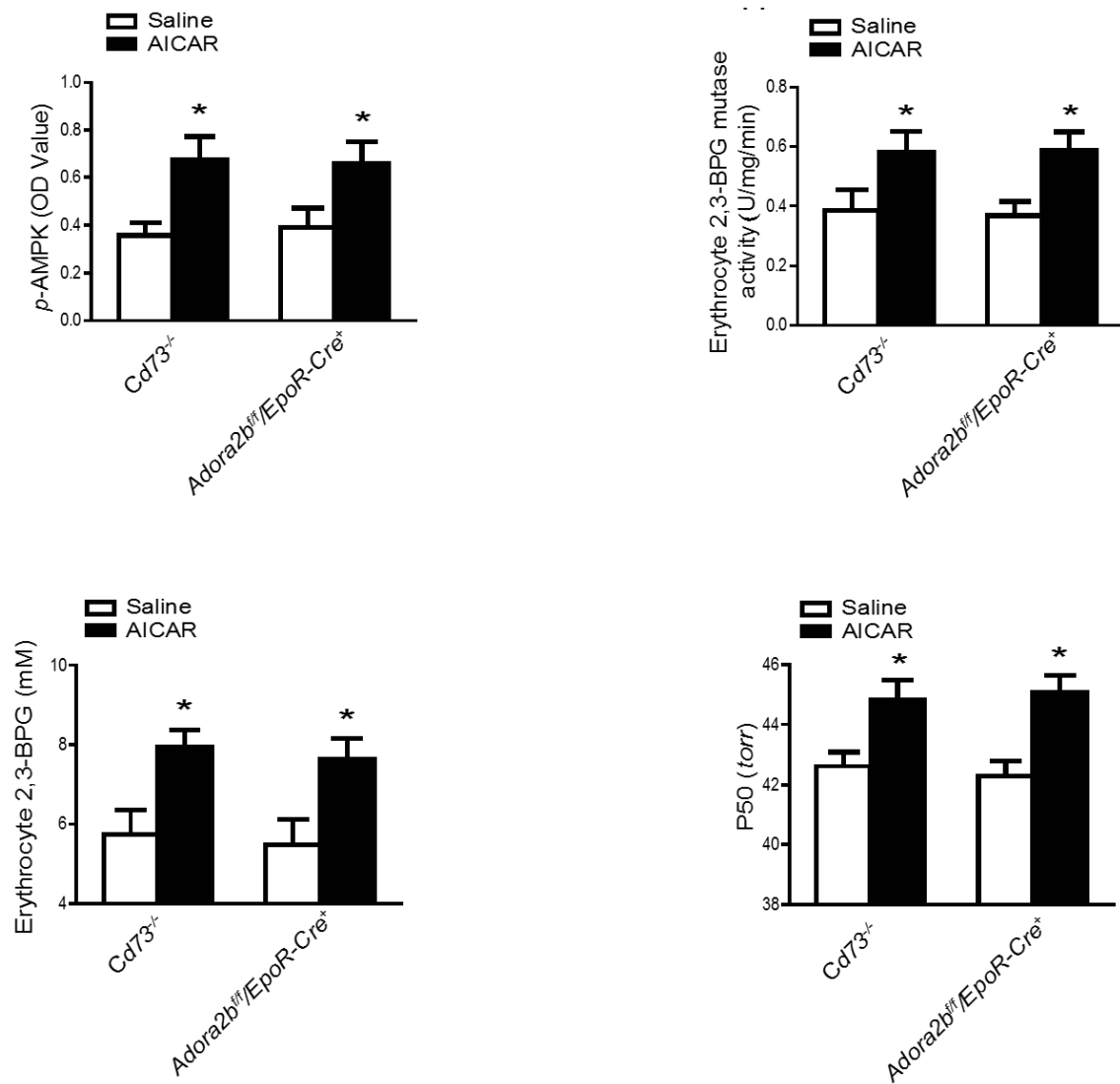


Figure 16. AICAR treatment significantly stimulated hypoxia-induced erythrocyte AMPK phosphorylation (A), 2,3-BPG mutase activity (B), 2,3-BPG production (C) and P50 levels (D) compared to saline-treated group in *Cd73^{-/-}* mice and *Adora2b^{fl}/EpoR-Cre⁺* mice under hypoxia (10% O₂, 90% N₂) for 3 days. Data are expressed as mean \pm SEM; **P*<0.05 for AICAR-treated mice vs saline-treated mice (n=8).

2.3.8 AMPK functions downstream of erythrocyte ADORA2B in mice and underlies hypoxia-induced 2,3-BPG production by phosphorylation and activation of 2,3-BPG mutase

Erythrocyte 2,3-BPG is synthesized from the glycolytic intermediate 1,3-BPG by 2,3-BPG mutase (Fig. 4A). Factors regulating 2,3-BPG production are poorly understood. A previous report provided evidence that 2,3-BPG mutase is associated with AMPK in erythrocyte lysates and may be a substrate of AMPK¹¹⁴. Additional studies showed that ADORA2B signaling activates AMPK in other cellular systems¹¹⁵. Like adenosine, AMPK plays a critical role in multiple cellular functions especially under conditions of energy depletion and limited O₂ availability¹¹⁶. However, whether AMPK functions downstream of ADORA2B as a key enzyme responsible for hypoxia-induced 2,3-BPG production in erythrocytes has not been previously studied. To test this possibility, we exposed *EpoR-Cre* mice and *Adora2b^{fl/fl}/EpoR-Cre⁺* mice to hypoxia (10% oxygen, similar to that found at 5260 m) for different time points. We found that erythrocyte AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG level and P50 are significantly induced in *EpoR-Cre* mice after 1 day, 3 days and 7 days hypoxia treatment. However, hypoxia induced elevation of AMPK phosphorylation, 2,3-BPG mutase/synthase activity, 2,3-BPG and P50 are attenuated in *Adora2b^{fl/fl}/EpoR-Cre⁺* mice after 1 day, 3 days and 7 days hypoxia treatment (Fig. 14A-D). These studies demonstrated that hypoxia is capable of inducing AMPK phosphorylation, subsequently activate 2,3-BPG mutase, generate 2,3-BPG and trigger oxygen release, and that erythrocyte ADORA2B is essential for this phenomenon *in vivo*.

Next, we conducted experiments to assess the interaction between erythrocyte AMPK and 2,3-BPG mutase and determine whether activated AMPK phosphorylates 2,3-BPG mutase in erythrocytes. First, our immunoblots show that basal AMPK phosphorylation levels, as judged by phosphorylation at threonine 172 (T172) of the subunit, in erythrocytes from *Adora2b^{ff}/EpoR-Cre⁺* mice and *EpoR-Cre⁺* mice under normoxia were similar (Fig. 15A-B). Phosphorylation of AMPK at T172 was significantly induced in erythrocytes of *EpoR-Cre⁺* mice following 1 week of hypoxia (10% O₂). In contrast, phosphorylation of AMPK was suppressed in *Adora2b^{ff}/EpoR-Cre⁺* mice (Fig. 15A-B). Then, using an antibody that recognizes phosphorylated AMPK (*p*-AMPK) substrates we showed that *p*-AMPK specifically phosphorylates 2,3-BPG mutase and that under normoxic conditions the levels of AMPK-phosphorylated 2,3-BPG mutase were similar in *Adora2b^{ff}/EpoR-Cre⁺* mice and *EpoR-Cre⁺* mice (Fig. 15C-D). Subsequently, we found that hypoxia significantly induced *p*-AMPK-phosphorylated 2,3-BPG mutase in erythrocytes of *EpoR-Cre⁺* mice and that this phosphorylation was significantly attenuated in *Adora2b^{ff}/EpoR-Cre⁺* mice (Fig. 15C-D). Thus, we conclude that ADORA2B-induced AMPK phosphorylation, followed by *p*-AMPK-phosphorylated 2,3-BPG mutase are key steps in the signaling pathway underlying hypoxia-induced 2,3-BPG production and O₂ release from erythrocytes to peripheral tissues in mice.

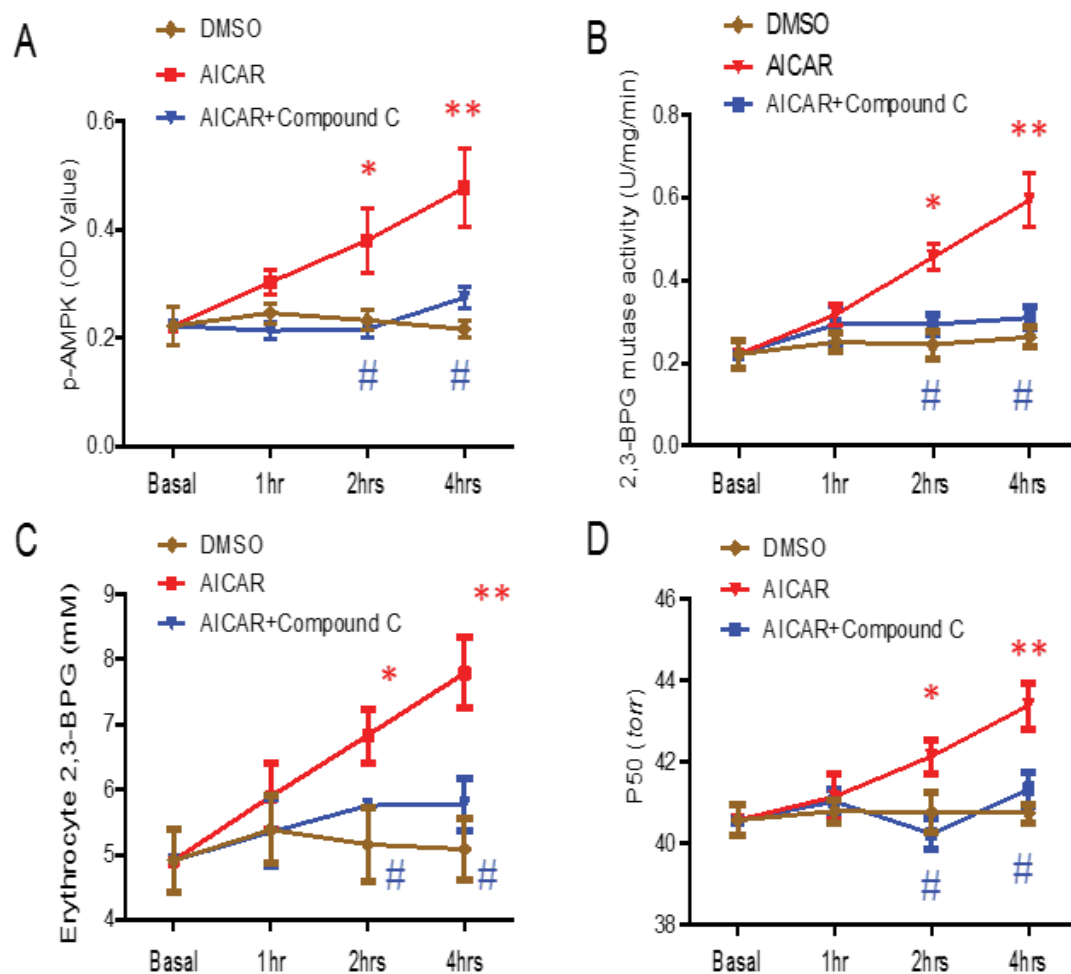


Figure 17. *In vitro* effects of AMPK activation in mouse erythrocytes.

(A-D) *In vitro* effects of AMPK activation. AMPK activator AICAR-induced p-AMPK (A), 2,3-BPG mutase activity (B), 2,3-BPG concentration (C) and P50 levels (D) in cultured erythrocytes isolated from WT mice in a time-dependent manner. While Compound C significantly attenuated effects of AICAR treatment. Data are expressed as mean \pm SEM; * $P < 0.05$ for AICAR-treated group 2-hour vs 1-hour, and for AICAR-treated group 2-hour vs DMSO-treated group 2-hour, ** $P < 0.05$ for AICAR-treated group 4-hour vs 2-hour, and for AICAR treated-group 4-hour vs DMSO-treated group 4-hour. # $P < 0.05$ for AICAR plus Compound C vs AICAR at the same time point (n=8).

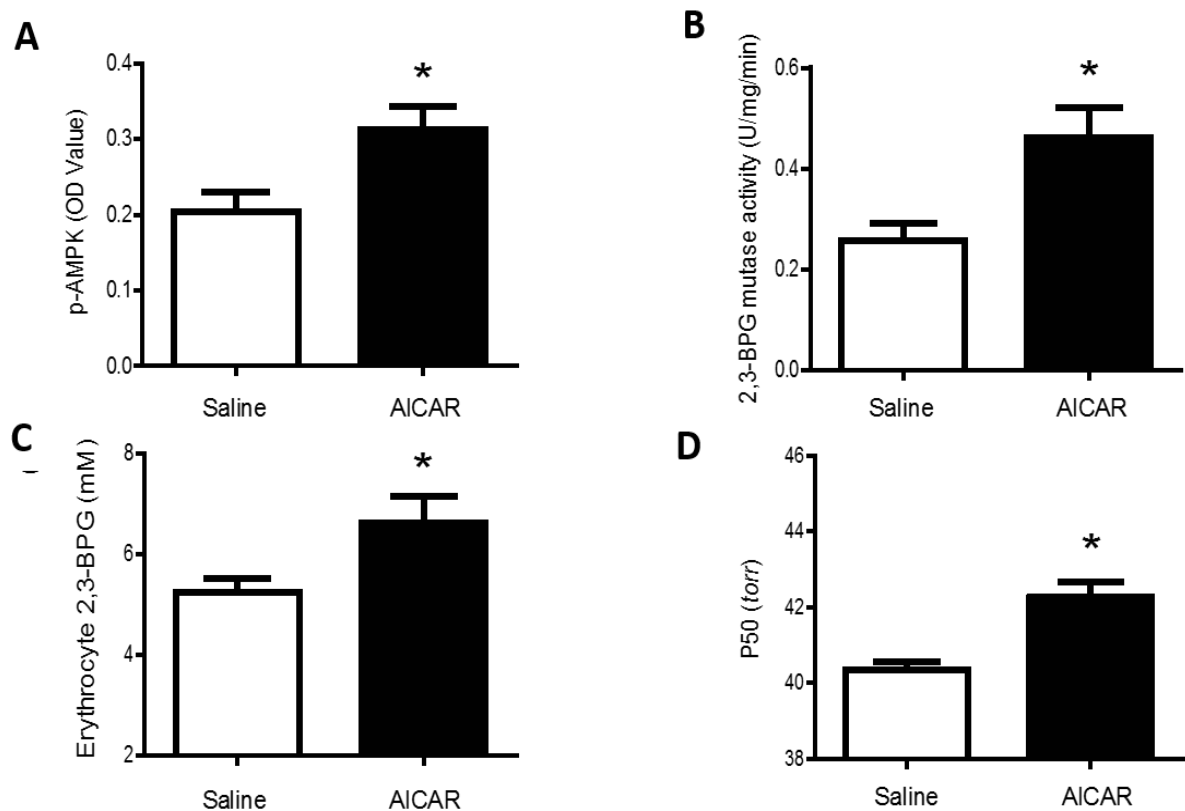


Figure 18. *In vivo* effects of AMPK activation in mouse erythrocytes.

(A-D) *In vivo* effects of AMPK activation in mouse under normoxia. (E-H) AICAR treatment (200mg/kg, IP) significantly stimulated erythrocyte *p*-AMPK (E), 2,3-BPG mutase activity (F), 2,3-BPG production (G) and P50 levels (H) in WT mice compared to saline-treated WT mice after 2 hours under normoxia. Data are expressed as mean \pm SEM; * P <0.05 Saline group vs AICAR-treated group (n=8).

2.3.9 AMPK activators induce erythrocyte 2,3-BPG mutase activity, 2,3-BPG production and O₂ release in mouse *in vitro* and *in vivo*

Our discovery that erythrocyte AMPK is phosphorylated and subsequently phosphorylates 2,3-BPG mutase in response to ADORA2B signaling suggests that activation of AMPK may be sufficient to induce 2,3-BPG production by activating 2,3-BPG mutase. To test this possibility, we conducted *in vitro* studies to determine if AMPK activation is sufficient to induce 2,3-BPG production and trigger oxygen release in cultured WT mouse erythrocytes. Initially, we found that two independent AMPK activators, AICAR (5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide) and AICAR, significantly induce phosphorylation of AMPK and activity of 2,3-BPG mutase in a time dependent manner (Fig. 5a-b). Next, we found that both AMPK activators significantly increased 2,3-BPG production and O₂ releasing capacity in cultured erythrocytes in a time dependent manner (Fig. 4c-d). Thus, *in vitro* studies demonstrated that AMPK activation is sufficient to induce 2,3-BPG production and enhanced O₂ release in mouse erythrocytes. However, Compound C (dorsomorphin), a potent and selective inhibitor of AMPK, significantly attenuated AICAR and AICAR mediated induction of AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG and O₂ releasing capacity in cultured mouse erythrocytes (Fig. 5a-d).

We extended our *in vitro* studies to further test if AICAR, a FDA approved drug, could induce erythrocyte 2,3-BPG and O₂ releasing capacity by stimulating erythrocyte AMPK phosphorylation and 2,3-BPG mutase activity in WT mouse *in vivo*. Our results (Fig. 5e-h) show that AICAR pretreatment significantly induced

erythrocyte AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG level and O₂ releasing capacity in WT mouse *in vivo*.

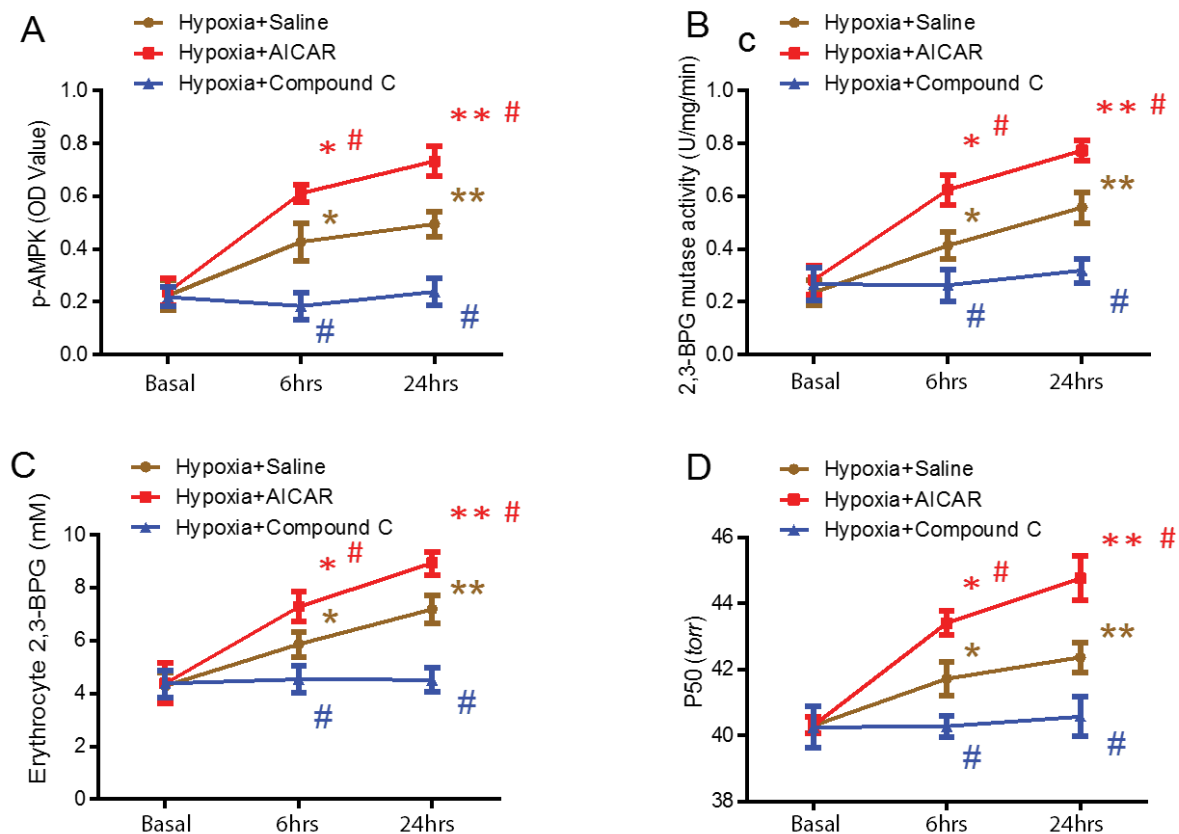


Figure 19. *In vivo* effects of AICAR and Compound C treatment under hypoxia.

(A-D) AICAR treatment significantly stimulated erythrocyte *p*-AMPK (A), 2,3-BPG mutase activity (B), 2,3-BPG production (C) and P50 levels (D) in WT mice compared to saline-treated WT mice under hypoxia (8% O₂) in a time-dependent manner, while Compound C treatment significantly attenuated erythrocyte *p*-AMPK (A), 2,3-BPG mutase activity (B), 2,3-BPG production (C) and P50 levels (D) in WT mice compared to saline-treated WT mice under hypoxia (8% O₂). Data are expressed as mean \pm SEM; **P*<0.05 for 6-hour vs basal level, ***P*<0.05 for 24-hour vs 6-hour. #*P*<0.05 for AICAR-treated group vs saline group, and Compound C-treated group vs saline group at the same time point (n=8).

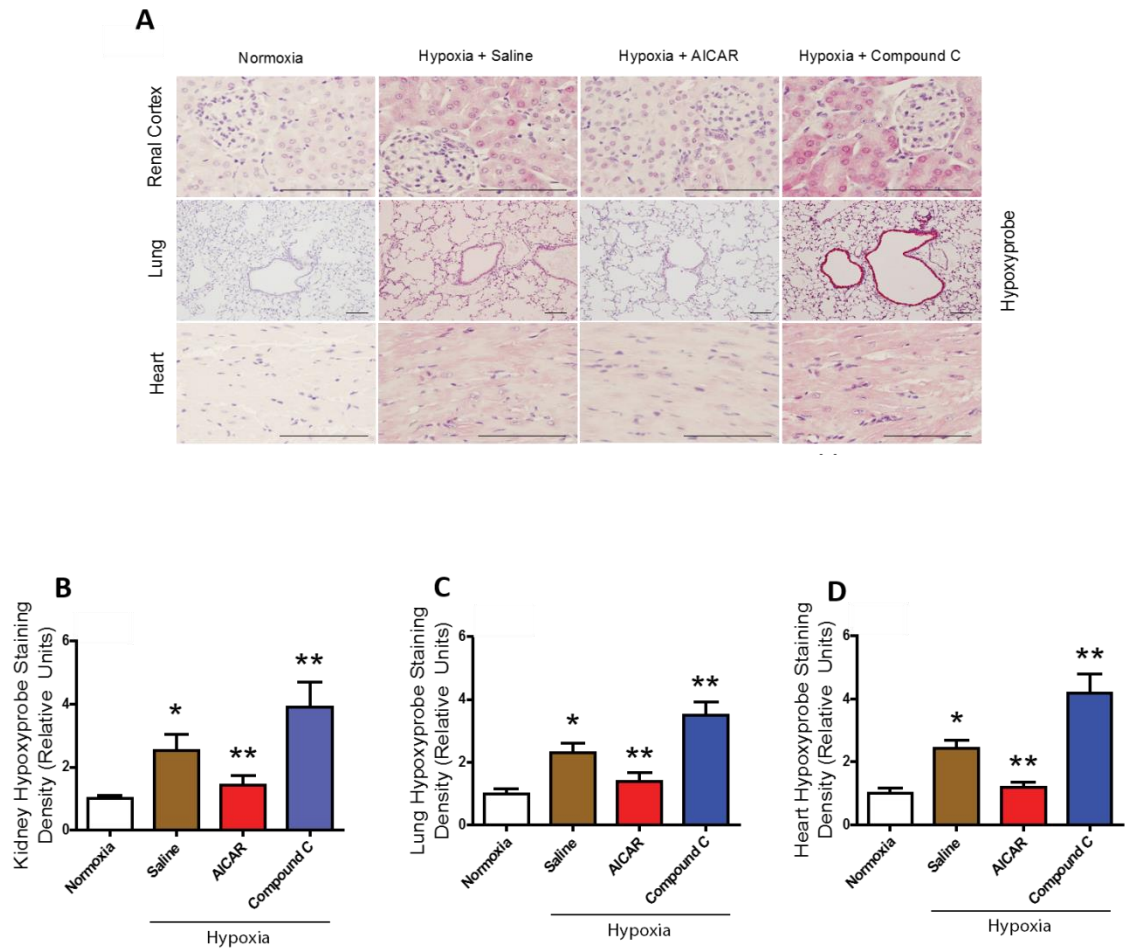


Figure 20. *In vivo* effects of AICAR or Compound C treatment on multiple tissues in mice under hypoxia. IHC analysis of tissue hypoxia by hypoxyprobe in kidney, lung and heart (**E**) Semi-quantification of the hypoxyprobe staining in multiple tissues including kidney, lung and heart of WT mice under 8% hypoxia condition for 24h with or without AICAR/Compound C treatment. Data are expressed as mean \pm SEM; * P <0.05 vs WT mice under normoxia condition; ** P <0.05 vs WT mice with saline treatment under hypoxia (n=8; Scale bar=200 μ M)

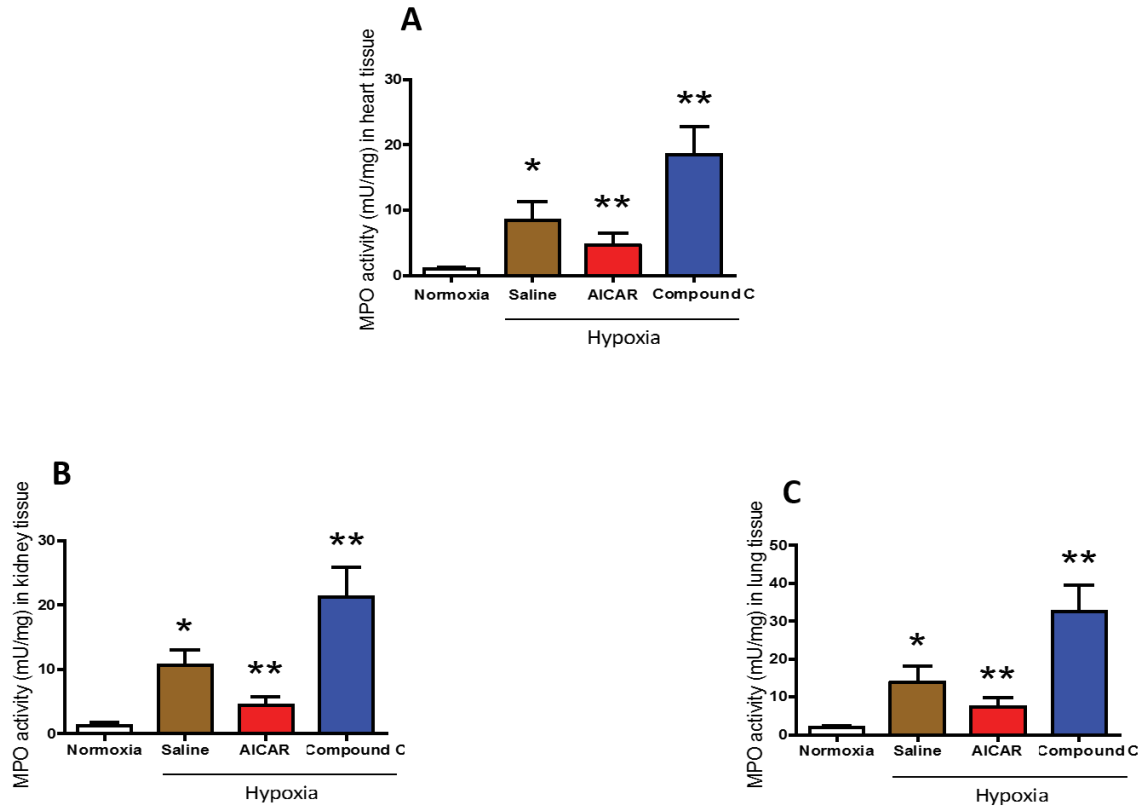


Figure 21. AICAR treatment prevented tissue inflammation infiltration, while Compound C treatment aggravated tissue MPO activity in WT mice under hypoxia for 24 hours.

MPO activity in heart (**A**), kidney (**B**) and lung (**C**). AICAR or Compound C-treated WT mice after 24 hours hypoxia treatment (n=8). Data are expressed as mean \pm SEM; * P <0.05 vs WT mice under normoxia; ** P <0.05 vs WT mice with saline treatment under hypoxia.

2.3.10 *In vivo* effects of AMPK agonist in hypoxia adaptation in mouse

To test whether AICAR could prevent hypoxia-induced multiple tissue damage in *Cd73^{-/-}* and *Adora2b^{fl/fl}/EpoR-Cre⁺* mice by stimulating erythrocyte 2,3-BPG production and O₂ releasing capacity, we treated these mice with AICAR under hypoxia (8% oxygen) for 3 days. Our results (Fig. 16A-D) show that AICAR treatment restored erythrocyte 2,3-BPG levels and P50 in both *Cd73^{-/-}* and *Adora2b^{fl/fl}/EpoR-Cre⁺* mice under hypoxia.

To determine the *in vivo* effect of AMPK agonist in hypoxia adaptation in WT mouse, we further extended our *in vivo* studies to treat WT mouse with AICAR under hypoxia (8% oxygen) up to 24 hours. We found that AICAR treatment induce greater elevation of erythrocyte 2,3-BPG and O₂ releasing capacity by further stimulating AMPK phosphorylation and 2,3-BPG mutase activity in WT mouse compared to saline group under hypoxia (Fig. 18A-D). Further histological results demonstrate that AICAR treatment significantly attenuated multiple tissue hypoxia including lungs, kidneys and hearts in WT mouse compared to saline group under hypoxia condition (Fig. 20A). Image quantification of hypoxyprobe staining demonstrated that hypoxic levels were significantly reduced by AICAR treatment in WT mouse (Fig. 20B-D). Next, to further determine the effect of AMPK inhibition in erythrocyte hypoxia adaptation, we treat WT mice with Compound C under hypoxia condition. In contrast, Compound C treatment reduce hypoxia-induced AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG levels and O₂ releasing capacity in WT mouse erythrocyte, thereby lead to enhanced multiple tissue hypoxia including lungs, kidneys and hearts compared to saline group under

hypoxia condition (Fig. 20A). Image quantification of hypoxyprobe staining demonstrated that hypoxic levels were significantly induced by Compound C treatment in WT mouse compared to saline group (Fig. 20B-D). Taken together, these studies demonstrate that AICAR can override a genetic block to hypoxia-induced adenosine production (i.e., CD73 deficiency) or erythrocyte ADORA2B signaling (erythrocyte-specific ADORA2B deficiency) to stimulate erythrocyte 2,3-BPG production and O₂ release by the pharmacologic activation of AMPK, and can facilitate earlier hypoxia adaptation in WT mouse by rapidly inducing erythrocyte AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG levels and subsequent O₂ release to peripheral tissues.

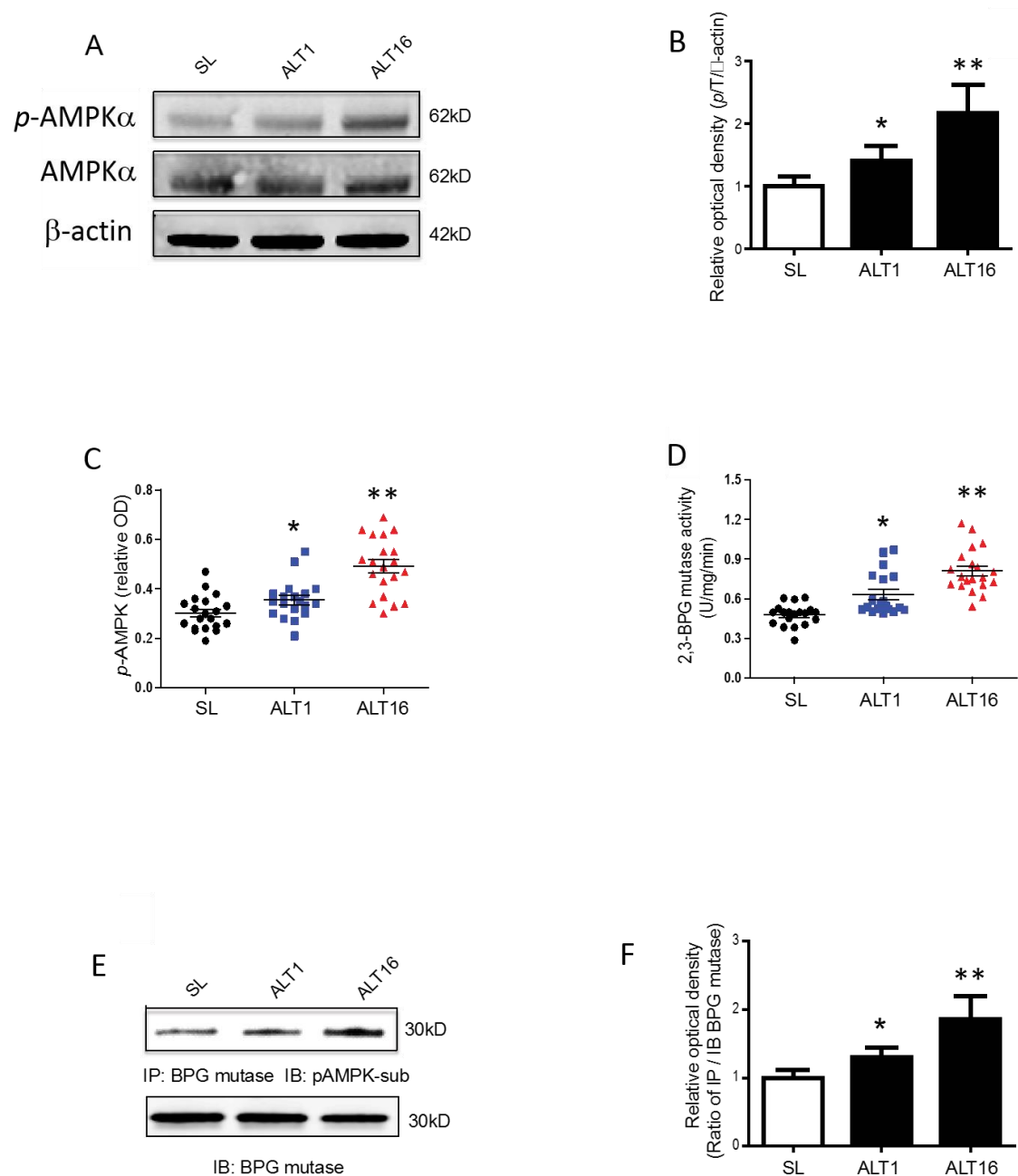


Figure 22. Erythrocyte *p*-AMPK and 2,3-BPG mutase activity are induced in humans at high altitude

(A and B) Representative western blot and relative image quantification analysis (n=3 per group) of *p*-AMPK levels quantified by ELISA C) and 2,3-BPG mutase

activity (D) at high altitude ALT1 and ALT16 over SL. (E and F) Representative western blot and relative image quantification analysis (n=3 per group) of *p*-AMPK phosphorylated 2,3-BPG mutase at SL and high altitude on day 1 and day 16 in erythrocyte lysates. Data are expressed as mean \pm SEM; * P <0.05 for high altitude on day 1 vs SL; ** P <0.05 for high altitude on day 16 vs day 1.

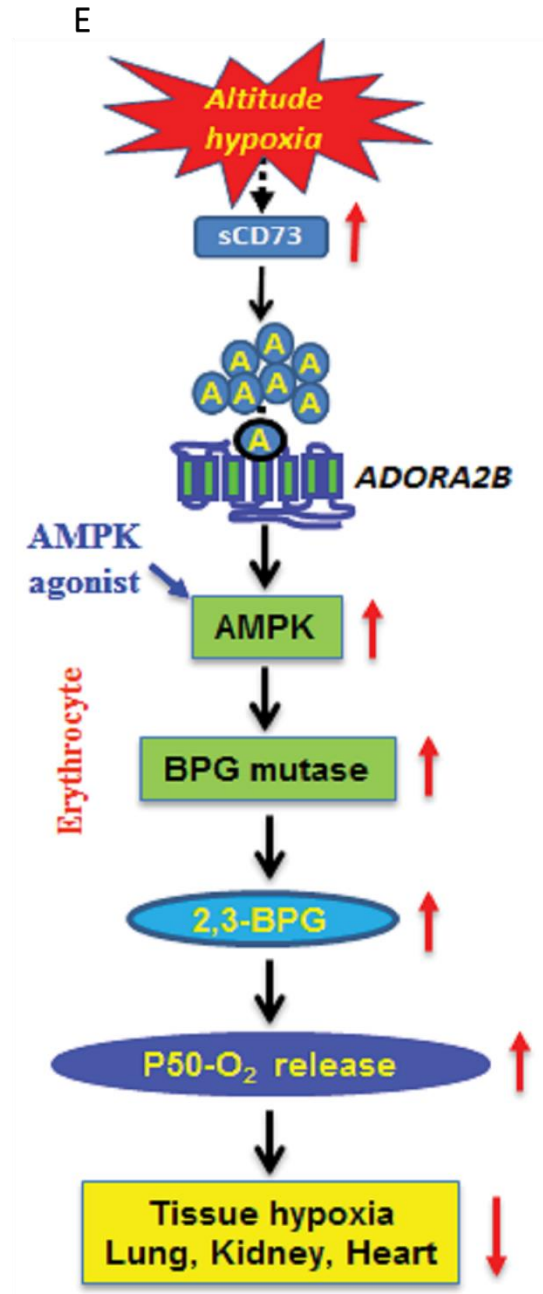
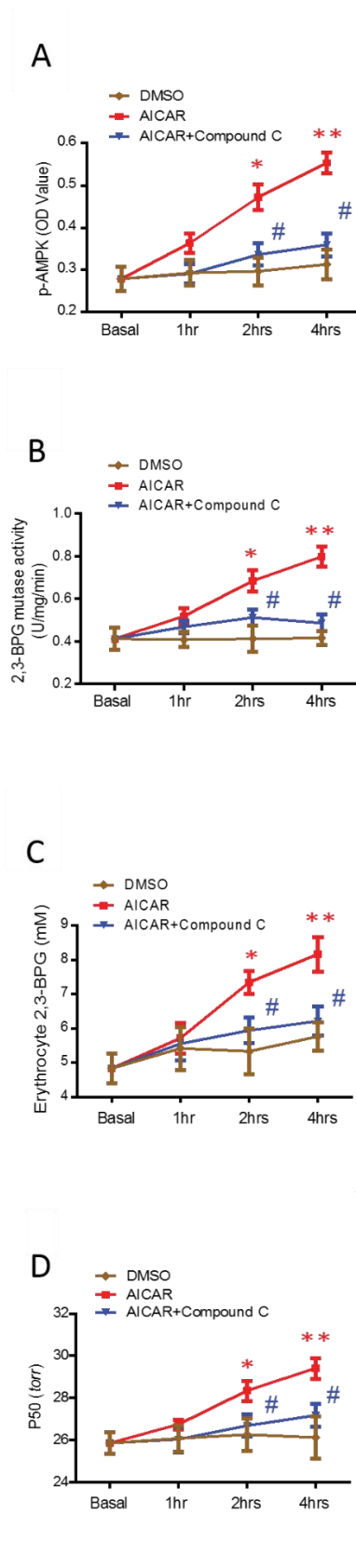


Figure 23. AMPK activation induces erythrocyte 2,3-BPG mutase activity, 2,3-BPG production and oxygen release in cultured human erythrocytes

(A-D) AMPK activator AICAR-induced phosphorylation of AMPK (A), 2,3-BPG mutase activity (B), 2,3-BPG concentration (C) and P50 levels (D) in cultured normal human erythrocytes under normoxia in a time-dependent manner. Compound C significantly attenuated the effect of AICAR treatment. Data are expressed as mean \pm SEM; * $P < 0.05$ for AICAR-treated 2-hour group vs 1-hour group, and for AICAR-treated 2-hour group vs DMSO-treated 2-hour group, ** $P < 0.05$ for AICAR-treated 4-hour group vs 2-hour group, and for AICAR-treated 4-hour group vs DMSO-treated 4-hour group. # $P < 0.05$ for AICAR plus Compound C vs AICAR at the same time point (n=8). (E) Working model: CD73 is essential for high altitude hypoxia-induced plasma adenosine production. Elevated plasma adenosine prevents hypoxia-induced tissue inflammation and damage by activating ADORA2B on erythrocytes to induce 2,3-BPG mutase activity, 2,3-BPG production, and subsequently increased P50 and increased O₂ release to peripheral hypoxic tissues. AMPK is a key enzyme that functions downstream of ADORA2B to activate 2,3-BPG mutase, promote 2,3-BPG production and O₂ release from erythrocytes. Thus, enhancing the CD73-ADORA2B-AMPK signaling

pathway is a promising therapeutic strategy to treat or prevent hypoxia-induced tissue damage.

2.3.11 AMPK activation-induced 2,3-BPG and O₂ release via stimulating 2,3-BPG mutase in human erythrocytes, and AMPK phosphorylation as well as 2,3-BPG mutase activity is significantly induced in humans at high altitude.

To determine the physiological function and significance of AMPK activation in humans, we conducted a series of *in vitro* studies using human red blood cells. First, AICAR and AICAR treatment directly induced AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG and subsequent O₂ release in cultured normal human erythrocytes in a time dependent manner (Fig. 23A-D). To further determine loss of function of AMPK activation in elevation of AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG production and O₂ release, we exposed AICAR and AICAR treated human normal RBCs with or without compound C. We found that compound C significantly suppressed AICAR and AICAR induced AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG levels and O₂ release in cultured human normal RBCs (Fig. 23A-D).

Next, we further investigate whether increased AMPK phosphorylation and 2,3-BPG mutase activity occurs in human erythrocytes at high altitude. We measured erythrocyte AMPK phosphorylation by western blot analysis in human subjects at sea level and at high altitude on ALT1 and ALT16. We found that erythrocyte *p*-AMPK levels were increased within two hours at high altitude (ALT1) and were further elevated following an extended stay at high altitude (ALT16) (Fig.

22A-B). In addition to western blotting we also used ELISAs to quantify erythrocyte *p*-AMPK levels and found that *p*-AMPK levels were significantly increased on ALT1 and further elevated on ALT16 (Fig. 22C). No significant differences in elevated *p*-AMPK levels were observed between male and female volunteers in response to high altitude (Fig. 6D). Additionally, we demonstrated that erythrocyte 2,3-BPG mutase activity were induced within two hours at high altitude (ALT1) and were further elevated following an extended stay at high altitude (ALT16) (Fig. 22D). Finally, we demonstrated that levels of AMPK-phosphorylated 2,3-BPG mutase in erythrocytes were significantly increased at ALT1, and further enhanced on ALT16 (Fig. 22E-F). Altogether, we demonstrated that 1) high altitude induces erythrocyte 2,3-BPG mutase activity; 2) two independent AMPK agonists induce AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG production and subsequent O₂ release in cultured human RBCs; 3) compound c inhibits AMPK activation and subsequently suppresses AICAR and AICAR induce AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG production and O₂ release in cultured human RBCs.

2.4 Discussion

It has been known for more than four decades that when humans ascend to high altitudes the affinity of Hb for O₂ is decreased to make more O₂ available to hypoxic peripheral tissues^{88, 117-119}. It was also recognized early on that the reduced O₂ affinity was due in part to the increase in erythrocyte 2,3-BPG which functioned as a negative allosteric regulator of Hb-O₂ affinity^{87, 120, 121}. The molecular mechanisms accounting for the altitude mediated regulation of erythrocyte 2,3-BPG concentrations were not understood until we conducted non-biased metabolomic screening of erythrocytes from a cohort of 21 human volunteers under normoxia and high altitude hypoxia. We report here that Rapoport-Luebering Shunt responsible for the production of erythrocyte 2,3-BPG is rapidly induced within two hours of arrival at 5260 m and increases further upon prolonged stay at that altitude. Additionally, we observed that plasma adenosine concentration and sCD73 activity increased along with erythrocyte 2,3-BPG levels and O₂ releasing capacity in humans at high altitude. Using genetic tools, we demonstrated for the first time that CD73-dependent elevation of plasma adenosine signaling via erythrocyte specific ADORA2B is a key mechanism for hypoxia adaptation by inducing 2,3-BPG production and triggering O₂ release to counteract multiple tissue severe hypoxia.

Mechanistically, we revealed that AMPK is a key enzyme that functions downstream of ADORA2B signaling and contributes to hypoxia-induced 2,3-BPG production and subsequent O₂ release from erythrocytes in mice and humans. Overall, both human and mouse studies reported here provide strong evidence that CD73-dependent elevation of plasma adenosine, signaling via ADORA2B on erythrocytes, plays a beneficial role in preventing altitude-mediated tissue hypoxia by inducing 2,3-BPG production and triggering O₂ release in a AMPK-dependent manner (Fig. 23E). Thus, our findings reveal novel therapeutic targets to protect against tissue hypoxia and provide a strong foundation for future clinical trials.

Although extracellular adenosine is known to be induced under hypoxia, no prior study has reported that circulating adenosine levels are increased at high altitude and a role of elevated adenosine in high altitude adaptation has never been recognized prior to our study with young healthy human volunteers. Here we discovered for the first time that plasma adenosine levels increased within two hours of arrival at high altitude and increased further upon prolonged stay at 5260 m. Consistent with the altitude-dependent increase in plasma adenosine, we also found that sCD73 activity was also significantly induced by high altitude. Moreover, we confirmed early studies showing that erythrocyte 2,3-BPG and O₂ releasing

capacity are significantly elevated in humans at high altitude. The elevated plasma adenosine concentrations is proportional to increased erythrocyte 2,3-BPG levels and O₂ releasing capacity. Although adenosine signaling regulates numerous cellular and tissue functions by engaging its membrane receptors^{113, 122}, the physiological function of adenosine and ADORA2B signaling in normal erythrocytes in response to hypoxia was unknown.

Similar to humans at high altitude, we found that sCD73 activity, circulating adenosine levels, erythrocyte 2,3-BPG levels and O₂ releasing capacity were significantly elevated in wild type mice maintained for 1 week at 10% O₂ (equivalent to the oxygen level at 5260 m). However, genetic deletion of CD73 significantly reduced hypoxia-induced plasma adenosine, erythrocyte 2,3-BPG levels and O₂ release capacity in the mice. The decreased O₂ availability to peripheral tissues resulted in multiple severely hypoxic tissues. Consistent with our findings, early studies showed that genetic deletion of CD73 leads to vascular leakage and increased immune cell infiltration in the lungs under hypoxic condition.^{48, 75} However, the functional role of CD73-dependent elevated circulating adenosine in the induction of 2,3-BPG levels and O₂ release capacity from normal erythrocytes to protect hypoxic tissue damage was not recognized prior to our studies.

Erythrocyte specific ADORA2B knockouts allowed us to demonstrate for the first time that ADORA2B is essential for elevated plasma adenosine induced-2,3-BPG production and O₂ releasing capacity from erythrocytes. The ablation of erythrocyte ADORA2B in mice results in severely hypoxic tissues, similar to CD73-deficient mice. Thus, we have identified that elevated plasma adenosine signaling via erythrocyte ADORA2B plays an important role in preventing multiple hypoxic tissues by inducing erythrocyte 2,3-BPG levels and triggering O₂ release to local tissues. The discovery of this erythrocytes signaling pathway reveals the importance of adenosine signaling in adaptation to high altitude hypoxia.

AMPK is a well-known metabolic stress-sensing kinase, which plays an essential role in regulating cellular energy metabolism.^{123, 124} In erythrocytes, AMPK plays critical roles in regulating oxidative stress and maintaining the integrity and life span of the cell^{101, 125-127}. A previous study used a proteomic approach to identify 2,3-BPG-mutase as a potential AMPK target in erythrocytes¹¹⁴. However, a role for AMPK in 2,3-BPG induction under high altitude or hypoxic conditions in normal erythrocytes has not been previously investigated. Our current work revealed that AMPK, functioning downstream of ADORA2B, directly phosphorylates and activates 2,3-BPG-mutase resulting in increased production of

2,3-BPG, thereby promoting O₂ release. Moreover, we demonstrated that two independent AMPK activators (AICAR and AICAR) induce phosphorylation and activation of 2,3-BPG mutase, 2,3-BPG production and O₂ release in cultured WT mouse erythrocytes. In contrast, Compound C, a potent and selective inhibitor of AMPK, significantly attenuated effects of AICAR and AICAR in cultured WT mouse erythrocytes. Because of the importance of AMPK in the induction of 2,3-BPG production in erythrocytes, we conducted preclinical studies and found that pretreatment with AICAR, a FDA-approved drug that has been used safely to treat patients with type 2 diabetes since 1957¹²⁸, prevented multiple severely hypoxic tissues both in CD73-deficient mice and erythrocyte specific ADORA2B-deficient mice by inducing erythrocyte 2,3-BPG levels and O₂ availability to local tissue. Moreover, we demonstrated that AICAR can induce earlier hypoxic adaptation by rapidly inducing erythrocyte 2,3-BPG levels, triggering O₂ release and thereby prevent tissue hypoxia in WT mice. However, loss of function of AMPK by Compound C treatment leads to suppression of erythrocyte AMPK phosphorylation, 2,3-BPG mutase activity, 2,3-BPG level and O₂ release capacity, thereby abolish effect of erythrocyte hypoxia adaptation resulting severe multiple tissue hypoxia. The significance of our mouse finding was further supported by human translational

studies showing that both AICAR and AICAR induce phosphorylation of AMPK and activation of 2,3-BPG mutase, 2,3-BPG production and O₂ release in cultured normal human erythrocytes. In contrast, Compound C attenuate AMPK activation-mediated AMPK phosphorylation, 2,3-BPG mutase activation, 2,3-BPG production and O₂ release in cultured normal human erythrocytes. Strikingly, AMPK phosphorylation, 2,3-BPG mutase activity and levels of *p*-AMPK-phosphorylated 2,3-BPG mutase are significantly elevated in the erythrocytes of humans under high altitude. Thus, preclinical studies in mice and translational studies with human volunteers provide a strong foundation for future clinical trials in humans to test the ability of AICAR to increase O₂ availability in humans adapting to high altitude or other hypoxic challenges. Moreover, future clinical trials to treat or prevent high altitude sickness or other disease involving hypoxia-induced tissue damage should be facilitated by the safety profile and FDA acceptance of AICAR (Fig. 23E).

In conclusion, we have identified a functional role of CD73-dependent elevated extracellular adenosine signaling in O₂ release from Hb in erythrocytes in response to hypoxia. This discovery has revealed a previously unrecognized role of adenosine signaling in erythrocyte physiology and provides a novel molecular mechanism underlying adaptation to high-altitude hypoxia. Moreover, our finding

that AMPK functioning downstream of ADORA2B in erythrocytes activates 2,3-BPG mutase, subsequently resulting in increased 2,3-BPG production and decreased Hb-O₂ binding affinity, has important clinical implications. Overall, our current studies have added significant insight to the molecular mechanisms underlying human adaptation to hypoxia and thereby have opened up novel therapeutic possibilities for prevention and treatment of hypoxia-related diseases.

3 Chapter 3 Role of adenosine signaling in erythroid progenitors

3.1 Introduction

Approximately 10^{11} new RBCs are produced daily through proliferation and differentiation of erythroid progenitors descent from hematopoietic stem cells (HSC). This so-called erythropoiesis is a dynamic process finely regulated by cytokines, hormones, growth factors, among others at transcriptional and translational level^{129, 130}. Stress-induced erythropoiesis is defined as a stimulated basal erythropoiesis with expansion of the erythroid progenitor pool, resulting in reticulocytosis and splenomegaly, in response to stress conditions including anemia, hypoxia or infection.

Cell differentiation and proliferation is largely regulated by energy metabolism. Although stress erythropoiesis has been long speculated to be linked with increased metabolic requirements¹³¹, it is only until the recent several years with innovative metabolomics profiling and state of art isotopically labelled metabolic flux approaches¹³², the importance of fuel resources including glucose and glutamine and their transport are revealed¹³³. However, the molecular basis promoting metabolic reprogramming to support erythropoiesis in response to stress condition remains largely unknown.

Insufficient oxygen availability is one of the major stimulus for stress erythropoiesis, inability to adapt to those stress conditions will result in multiple-tissue damage, stroke, cardiovascular dysfunction and even death^{89, 90, 134}. Previous studies have showed that adenosine signaling, a well-known key regulator in response to stress condition, is involved in erythropoiesis¹³⁵. However,

whether adenosine signaling contributes to stress erythropoiesis is unknown.

Here, our mouse genetic studies showed that activation of adenosine signaling via erythroid ADORA2B promotes the expansion of erythroid progenitors both in spleen and bone marrow and in this way contributes to stress erythropoiesis in two independent *in vivo* mouse models including hypoxia and PHZ-induced anemia. Follow-up unbiased high-throughput metabolic profiling identified that erythroid ADORA2B contributes to an overall hypoxia metabolic reprogramming with substantial increased glycolysis, TCA cycle intermediates production in erythroid progenitors in mice. Mechanistically, we showed that hypoxia-induced factor 1 alpha (HIF-1 α) functions as downstream of erythroid ADORA2B regulating hypoxia metabolic reprogramming by regulation of glucose and glutamine metabolism and their transporters, subsequently modulates commitment of an HSC to erythroid lineage. Taken together, our studies identify that adenosine erythroid ADORA2B is a previously unrecognized purinergic signaling underlying stress erythropoiesis by regulation of metabolic reprogramming in erythroid progenitors, and likely discover novel therapeutic targets to counteract stress-induced injury by induction of erythropoiesis.

3.2 Methods and material

3.2.1 Mouse Subjects

Eight to ten-week-old C57BL/6 wild-type (WT) mice were purchased from Harlan Laboratories (Indianapolis, IN). Ecto-5'-nucleotidase (CD73)-deficient mice and A2B adenosine receptor (ADORA2B)-deficient mice with C57BL/6 background were generated and genotyped as described before⁹⁴. A novel line of mice with erythrocyte specific deletion of *Hif-1 α* was generated by crossing mice homozygous for a floxed *Hif-1 α* allele with mice expressing Cre recombinase under the control of erythropoietin receptor (EpoR) gene regulatory elements⁹⁵. All protocols involving animal studies were reviewed and approved by the Institutional Animal Welfare Committee of the University of Texas Health Science Center at Houston.

3.2.2 Plasma Epo measurement

Blood was collected with heparin as an anti-coagulant, and plasma were collected by centrifugation at 2000 x g for 20 min at room temperature. Then the plasma Epo concentration was measured by using commercial ELISA kits. (R&D System, Inc.)

3.2.3 Flow cytometry

For flow cytometry analyses, single cell suspensions were first prepared from blood, spleen, or bone marrow. $\sim 5 \times 10^5$ cells were washed with PBS/2%FBS (wash buffer), then incubated with 1-2 μ g/ml indicated antibodies 30mins at 4°C. Mouse antibodies used including CD11b-APCCy7, CD71-PECy7, Gr1-APCCy7, Ter119-Pacific blue (all from Biolegend). Cells were additionally stained with matching

isotype controls. For viability assessments, cells were stained with annexin V-APC in conjunction with 7-AAD. Cells were then washed with wash calcium binding buffer. Data was acquired with a Beckman-Coulter Gallios Flow Cytometer and analyzed with Kaluza1.5 software as previously¹³⁶.

3.2.4 RT-PCR

RNA extraction, reverse transcription and real time-PCR were performed as previously described^{97, 108}. SYBR green was used for analysis of all the genes, and the relative RNA levels were quantified as the $\Delta\Delta CT$ method¹⁰⁹. Primers: Mouse Adora1: forward; 5'-TGTGCCCCGGAATGTACTGG-3' and reverse; 5'-TCTGTGGCCCAATGTTGATAAG-3', Mouse Adora2a: forward; 5'-GCCATCCCATTGCGCCATCA-3' and reverse; 5'-GCAATAGCCAAGAGGCTGAAGA-3', Mouse Adora2b: forward; 5'-GCGAGAGGGATCATTGCTG-3' and reverse; 5'-CAGGAACGGAGTCAATCCAA-3', Mouse Adora3: forward; 5'-ATACCAGATGTCGCAATGTGC-3' and reverse; 5'-GCAGGCGTAGACAATAGGGTT-3', Mouse Gapdh: forward; 5'-TGACCTCAACTACATGGTCTACA-3' and reverse; 5'-CTTCCCATTCTCGGCCTTG-3'; Mouse HIF-1 α : forward; 5'-GAAATGGCCCAGTGAGAAAA-3' and reverse; 5'-CTTCCAVGTTGCTGACTTGA-3'.

3.2.5 Mouse bone marrow stem cell culture *ex-vivo*

Bone marrow cells (BMCs) from WT mice and *Adora2b*^{-/-} were collected as described in Chapter 2. Cultured cells were harvested at 0, 24h and 48h time points and then detected by flow cytometry^{106, 107}.

3.3 Results

3.3.1 Elevated CD73-mediated plasma adenosine is essential for hypoxia-induced erythroid commitment and erythropoiesis in mice independent of plasma erythropoietin

To determine whether adenosine contributes to the hypoxia-induced stress erythropoiesis, we exposed wild type mice (WT) and CD73-deficient mice (*Cd73*^{-/-}) to hypoxia (10% O₂) up to 72h. We found that the soluble CD73 (sCD73) activity and levels of plasma adenosine were induced after hypoxia exposure in WT mice in a time-dependent manner (Fig. 24A-B). Moreover, erythropoiesis-related parameters including percentage of reticulocyte and spleen size were significantly induced in response to hypoxia compared to normoxia in WT mice (Fig. 24D-E). However, hypoxia-mediated elevation of sCD73, increased plasma adenosine level and induction of erythropoiesis were significantly suppressed in *Cd73*^{-/-} mice compared to WT mice. In contrast, plasma erythropoietin (Epo) levels were no significant difference between WT and *Cd73*^{-/-} mice under normoxia or hypoxia condition (Fig. 24A-E). Erythropoiesis was detected by flow cytometry in WT mice including increased percentage of splenic CD71⁺ and Ter119⁺ erythroid progenitors in response to hypoxia compared to normoxia, with a similar trend in bone marrow

(Fig. 25A). To further evaluate erythroid lineage differentiation, I assessed the Ter119⁺ cells on the basis of CD71 staining from stage I to stage IV which represents undifferentiated cells to differentiated cells (Fig. 25B). Hypoxia-induced expansion of erythroid progenitors was induced most in the stage II of erythroid progenitors in WT mice, but was suppressed in *Cd73*^{-/-} mice. In contrast, attenuated myeloid lineage (CD71⁻CD11b⁺, CD71⁻Gr1⁺) was detected in bone marrow in WT mice as compared to *Cd73*^{-/-} mice after hypoxia exposure (Fig. 25C). Thus, here I provide genetic evidence that CD73-mediated increased plasma adenosine is required for hypoxia-induced erythroid commitment and erythropoiesis independent of plasma Epo level.

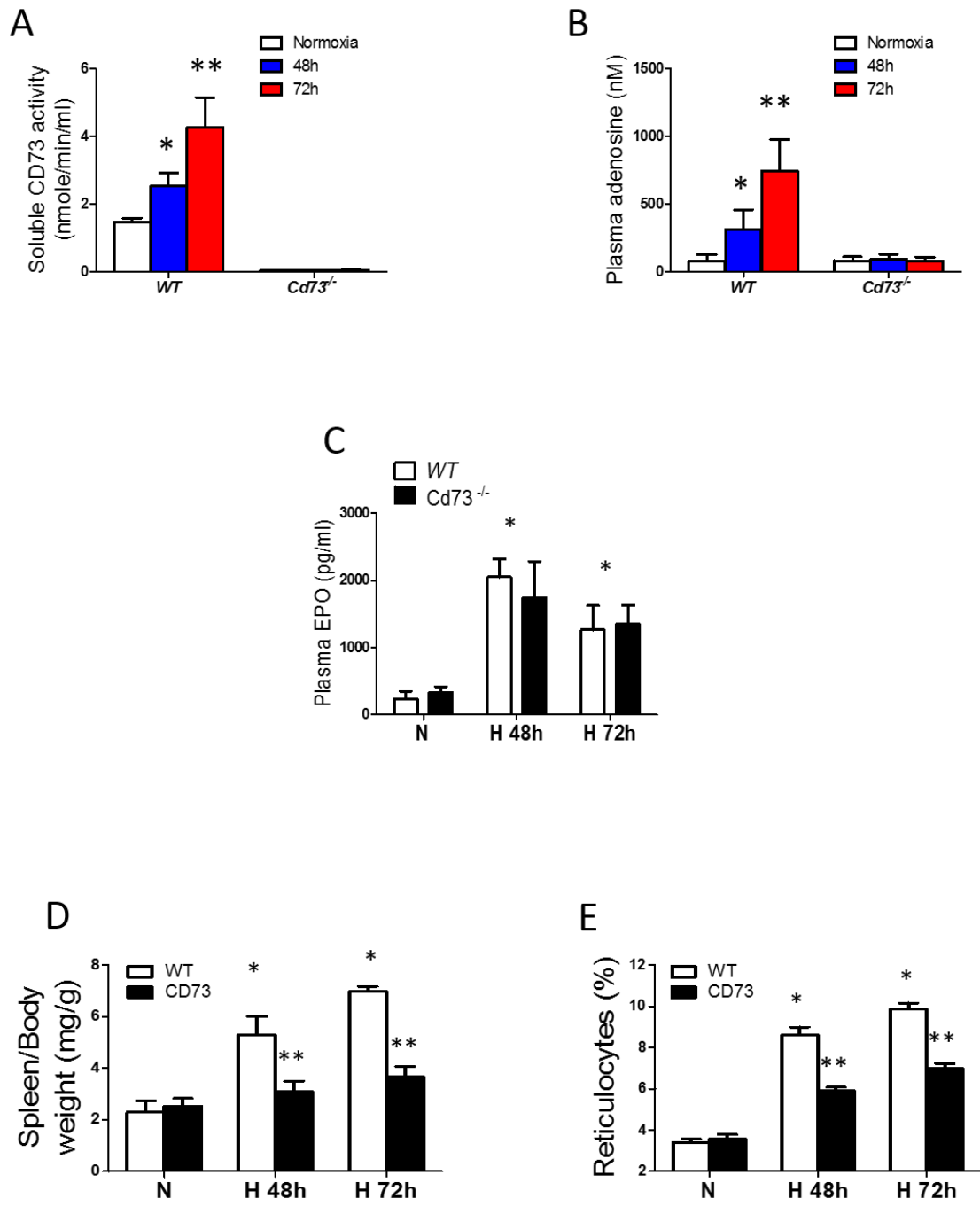


Figure24. Elevated CD73-mediated adenosine induction is essential for hypoxia-induced erythropoiesis in mice independent of plasma erythropoietin

(A-C) Soluble CD73 (sCD73) activity and levels of plasma adenosine were induced after hypoxia exposure in WT mice, but not in *Cd73*^{-/-} mice in a time-dependent manner. Data are expressed as mean \pm SEM; **P*<0.05 vs WT under normoxia; ***P*<0.05 vs WT under 48h hypoxia (n=10) (C) No difference of elevated erythropoietin (Epo) level was detected between WT and *Cd73*^{-/-} mice under normoxia or hypoxia condition. Splenomegaly (D) and percentage of reticulocyte (E) and were significantly induced in response to hypoxia compared to normoxia in WT mice, but not in *Cd73*^{-/-} mice in a time-dependent manner. Data are expressed as mean \pm SEM; **P*<0.05 vs WT under normoxia; ***P*<0.05 *Cd73*^{-/-} vs WT under hypoxia (n=10).

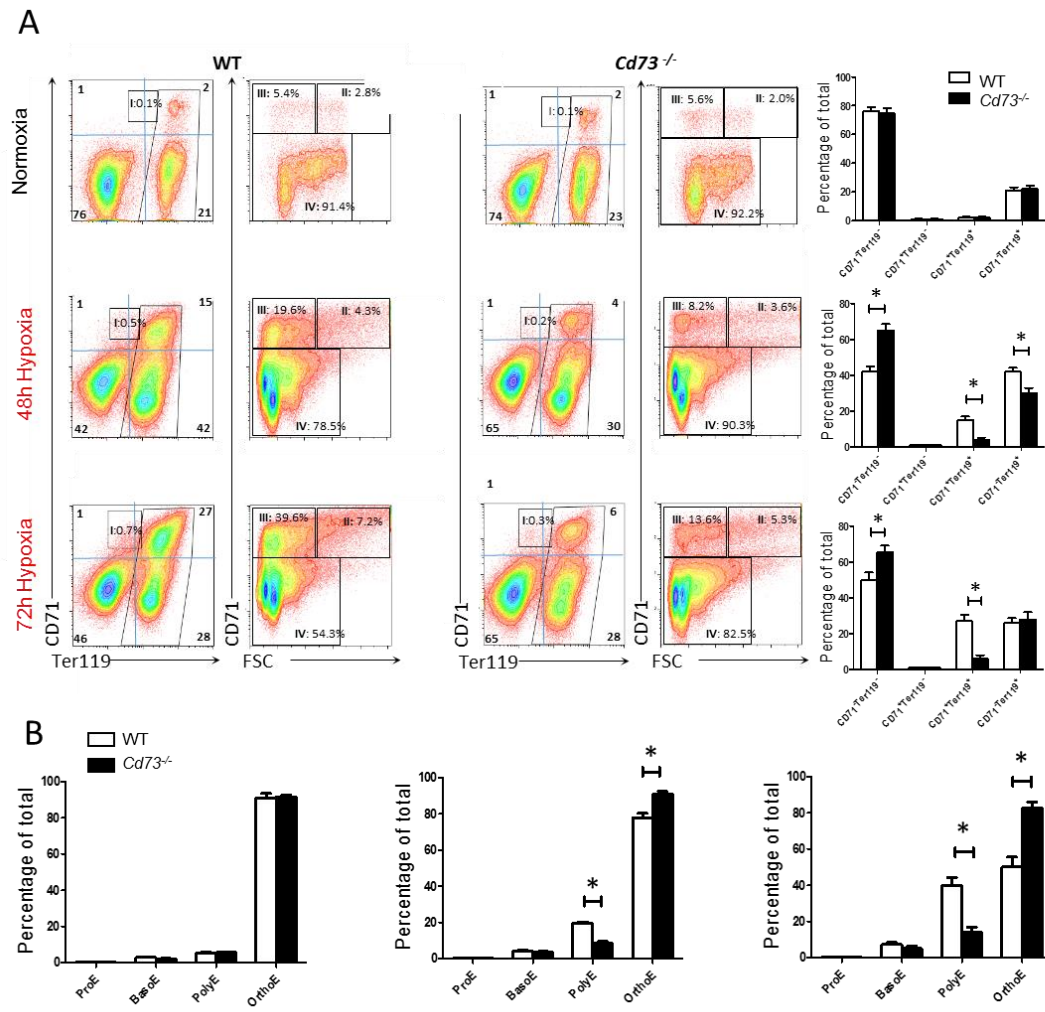


Figure. 25. Elevated CD73-mediated adenosine induction is essential for hypoxia-induced erythroid expansion in mice

(A) Robust erythropoiesis was detected by flow cytometry in WT mice including increased percentage of splenic CD71⁺ or Ter119⁺ or CD71⁺/Ter119⁺ erythroid progenitors in response to hypoxia compared to normoxia. (B) Erythroid lineage differentiation, reflected by the assessment of Ter119⁺ cells on the basis of CD71 staining from stage I to stage IV. Hypoxia-induced expansion of erythroid progenitors was induced the most in the stage II of erythroid progenitors in WT mice, but was suppressed in *Cd73*^{-/-} mice. (n=10). Data are expressed as mean \pm SEM.

3.3.2 Adenosine signaling contributes to stress erythropoiesis via erythroid ADORA2B

There are four GPCR adenosine receptors including ADORA1, ADORA2A, ADORA2B, and ADORA3. To determine which adenosine receptor is primarily involved in elevated plasma adenosine-mediated stress erythropoiesis in response to hypoxia, we specifically isolated the stage II of CD71⁺Ter119⁺ splenic erythroids, and subsequently assessed the adenosine receptor expression level by qRT-PCR. We found that *Adora2b* was highly expressed in the CD71⁺Ter119⁺ erythroids compared to the other three adenosine receptors and that *Adora2b* gene expression was induced the most in these cells under hypoxia compared to normoxia (Fig. 26A), implicating that ADORA2B is likely the major adenosine receptor underlying elevated plasma adenosine-induced erythropoiesis under hypoxia. To test this hypothesis, we exposed *EpoR-Cre*⁺ mice (control) and *Adora2b*^{fl/fl}*EpoR-Cre*⁺ mice (erythroid specific ablation of *Adora2b* genes) to normoxia or hypoxia (10% O₂) up to 72h. 10% O₂ hypoxia induced similar levels of plasma adenosine in *EpoR-Cre*⁺ mice and *Adora2b*^{fl/fl}*EpoR-Cre*⁺ mice. However, hypoxia-induced erythropoiesis featured with increased percentage of reticulocyte and splenomegaly were significantly attenuated in *Adora2b*^{fl/fl}*EpoR-Cre*⁺ mice compared to *EpoR-Cre*⁺ mice (Fig. 26B-C). In addition, genetic deletion of erythroid ADORA2B significantly attenuated hypoxia-induced population of stage II-CD71⁺Ter119⁺ erythroids in *Adora2b*^{fl/fl}*EpoR-Cre*⁺ mice compared to *EpoR-Cre*⁺ mice. Similar trend were observed in the bone marrow (Fig. 27A). In contrast, Epo levels were elevated to a similar extent in *EpoR-Cre*⁺ mice and *Adora2b*^{fl/fl}*EpoR-Cre*⁺ mice under hypoxia (Fig. 26D). Furthermore, attenuated myeloid lineage

(CD71⁺CD11b⁺, CD71⁺Gr1⁺, Ter119⁺CD11b⁺, Ter119⁺Gr1⁺) was detected in bone marrow of *EpoR-Cre*⁺ mice as compared to *Adora2b*^{fl/fl}*EpoR-Cre*⁺ mice (Fig. 27B-E). Thus, I provides genetic evidence that adenosine signaling via erythroid ADORA2B contributes to hypoxia-induced erythropoiesis by regulation of erythroid commitment of HSCs independent of plasma Epo level.

3.3.3 ADORA2B is critical for commitment of hematopoietic precursors to erythroids by regulation of metabolic reprogramming in response to stress condition

Metabolic pathways and their regulation has been reported to play an essential role in proliferating cells. Therefore, stress erythropoiesis with substantially stimulated proliferation and differentiation of erythroid progenitors has been speculated to be linked with increased metabolic requirements, and nutrient including glucose and glutamine were considered as essential supply for the erythroid commitment from hematopoietic stem cells.

Although we showed that erythroid ADORA2B is the specific receptor underlying adenosine-mediated expansion of erythroid progenitors and subsequent contribution of erythropoiesis in response to hypoxia, the intracellular mechanism is still unclear. Therefore, to explore the mechanism underlying adenosine ADORA2B-induced expansion of erythroid cells under stress condition, I conducted unbiased high throughput metabolomic screening in CD71^{high}/Ter119^{high} progenitors isolated from spleen of *EpoR-Cre*⁺ mice and *Adora2b*^{fl/fl}*EpoR-Cre*⁺ mice under normoxia and 72 hour hypoxia. Overall, 233 metabolites were identified and quantified out of over 9000 detected features (mass to charge ratios) in these cells. Analysis of these metabolic changes

revealed that glucose metabolism including glycolysis, pentose phosphate pathway (PPP), glutamine metabolism and glutathione pathway are the most induced in control mice under hypoxia. In contrast, hypoxia induced glycolysis, TCA cycle intermediates and glutaminolysis were significantly suppressed in *Adora2b^{ff}EpoR-Cre⁺* mice. Thus, our metabolomic profiling demonstrated for the first time that ADORA2B has a previously unrecognized role in "hypoxic metabolic reprogramming" to enhance glucose and glutamine metabolism thus promote stress erythropoiesis.

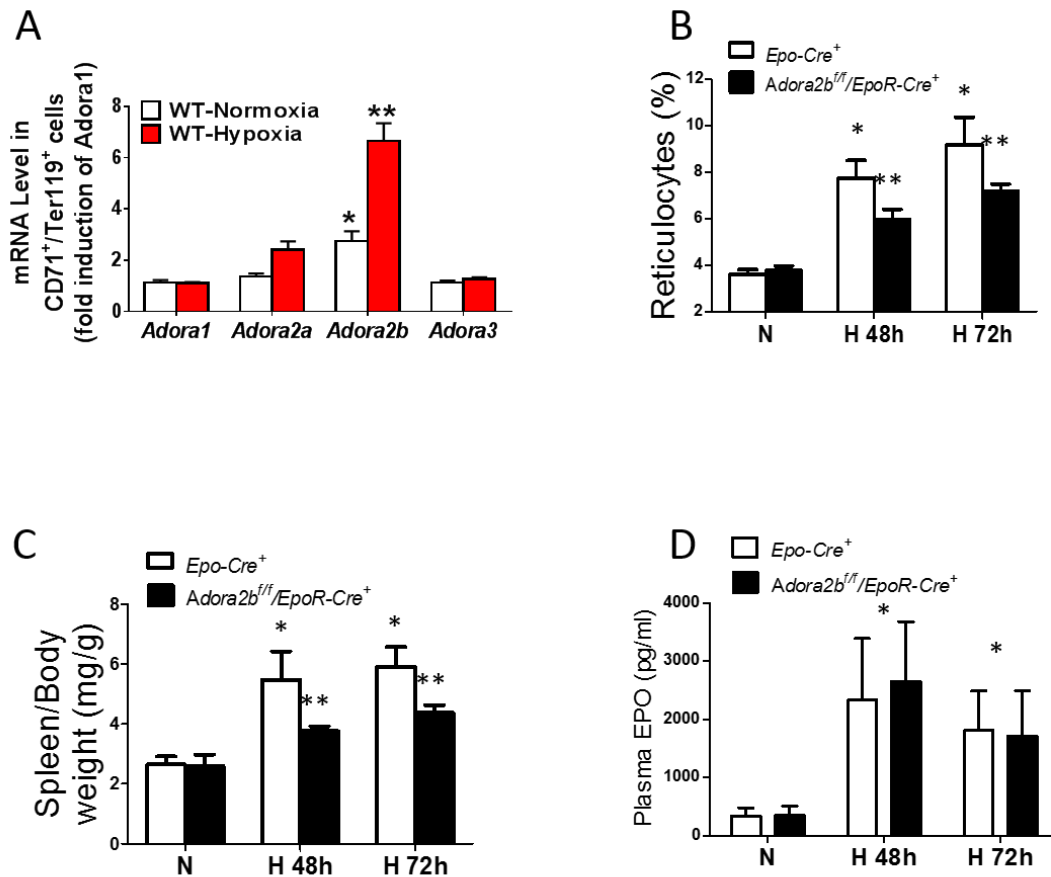


Figure. 26. Adenosine signaling via ADORA2B on erythroid progenitors contributes to stress erythropoiesis

(A) Four adenosine receptors including ADORA1, ADORA2A, ADORA2B, and ADORA3 *Adora2b* were assessed in the isolated CD71⁺Ter119⁺ splenic erythroids under normoxia and 72h hypoxia. (B-C) Hypoxia-induced erythropoiesis featured with increased percentage of reticulocyte and splenomegaly in *EpoR-Cre*⁺ mice, was suppressed in *Adora2b*^{fl/fl}/*EpoR-Cre*⁺ mice. (D) Similar plasma Epo levels in *EpoR-Cre*⁺ mice and *Adora2b*^{fl/fl}/*EpoR-Cre*⁺ mice under hypoxia. Data are expressed as mean \pm SEM; **P*<0.05 vs *EpoR-Cre*⁺ under normoxia; ***P*<0.05 *EpoR-Cre*⁺ vs *Adora2b*^{fl/fl}/*EpoR-Cre*⁺ under hypoxia (n=10).

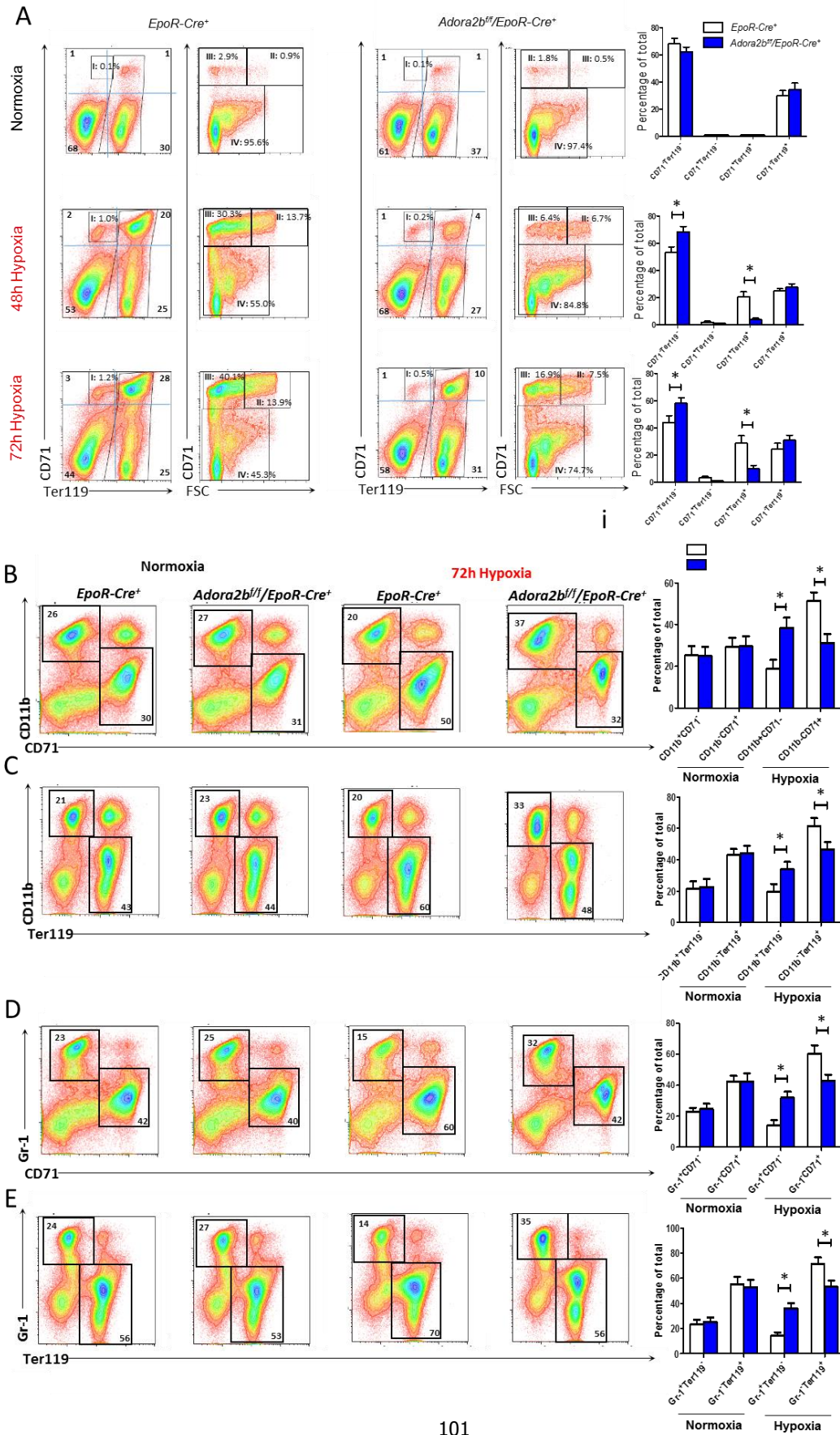


Figure. 27. Adenosine signaling via ADORA2B on erythroid progenitors regulates erythroid commitment under hypoxia

(A) Genetic deletion of erythroid ADORA2B significantly attenuated hypoxia-induced population of stage II-CD71⁺Ter119⁺ erythroids in *Adora2b^{fl/fl}EpoR-Cre⁺* mice compared to *EpoR-Cre⁺* mice. (B-E) Enhanced erythroid lineage (CD71⁺CD11b⁻, CD71⁺Gr1⁻, Ter119⁺CD11b⁻, Ter119⁺Gr1⁻), and attenuated myeloid lineage (CD71⁻CD11b⁺, CD71⁻Gr1⁺, Ter119⁻CD11b⁺, Ter119⁻Gr1⁺) were detected in *EpoR-Cre⁺* mice as compared to *Adora2b^{fl/fl}EpoR-Cre⁺* mice after 72h hypoxia exposure. Data are expressed as mean \pm SEM; **P*<0.05 vs *EpoR-Cre⁺* under normoxia; ***P*<0.05 *EpoR-Cre⁺* vs *Adora2b^{fl/fl}EpoR-Cre⁺* under hypoxia (n=10).

3.3.4 Erythroid HIF-1 α underlies ADORA2B contributes to hypoxia-induced stress erythropoiesis by regulation of erythroid commitment

How adenosine erythroid ADORA2B signaling regulates metabolic reprogramming, and subsequent control of the commitment of erythroid lineage from HSCs is still unknown. Previous study showed that HIF-1 α activation increases *Adora2b* gene expression under hypoxia. More recently, multiple studies showed that ADORA2B feedback induces *Hif-1* gene expression in other cellular systems. Further, RNA deep sequencing revealed that glucocorticoid-induced proliferation of erythroid progenitor cells was largely associated with HIF-1 α activation. Thus, I hypothesize that HIF-1 α and ADORA2B reciprocally upregulate each other in erythroid progenitor to promote stress erythropoiesis. To test this hypothesis, we conducted RT-PCR to show that *Hif-1* mRNA is significantly induced in these progenitor cells isolated from *EpoR-Cre*⁺ mice under hypoxia, but not in *Adora2b*^{f/f}*EpoR-Cre*⁺ mice, indicating that ADORA2B induces *Hif-1* gene expression under hypoxia in erythroid progenitor cells (Fig. 28A). Next, to determine the functional role of HIF-1 α in hypoxia-induced erythropoiesis, we generated *Hif-1* α ^{f/f}*EpoR-Cre*⁺ erythroid-specific HIF-1 α knockouts by mating *Hif-1* α ^{f/f} mice with *EpoR-Cre*⁺ mice. Intriguingly, we found robust hypoxia-induced stress erythropoiesis reflected by increased spleen size and percentage of reticulocyte in *EpoR-Cre*⁺ mice control mice, was suppressed in *Hif-1* α ^{f/f}*EpoR-Cre*⁺ mice (Fig. 28B-C). Moreover, we found that genetic deletion of erythroid HIF-1 α significantly reduced population II of CD71⁺Ter119⁺ proerythroblast progenitors in spleen compared to *EpoR-Cre*⁺ control mice after 72 hour hypoxia exposure

(Fig. 28D). Taken together, this provides genetic and pharmacological evidence that ADORA2B-HIF-1 α pathway contributes to hypoxia-induced stress erythropoiesis.

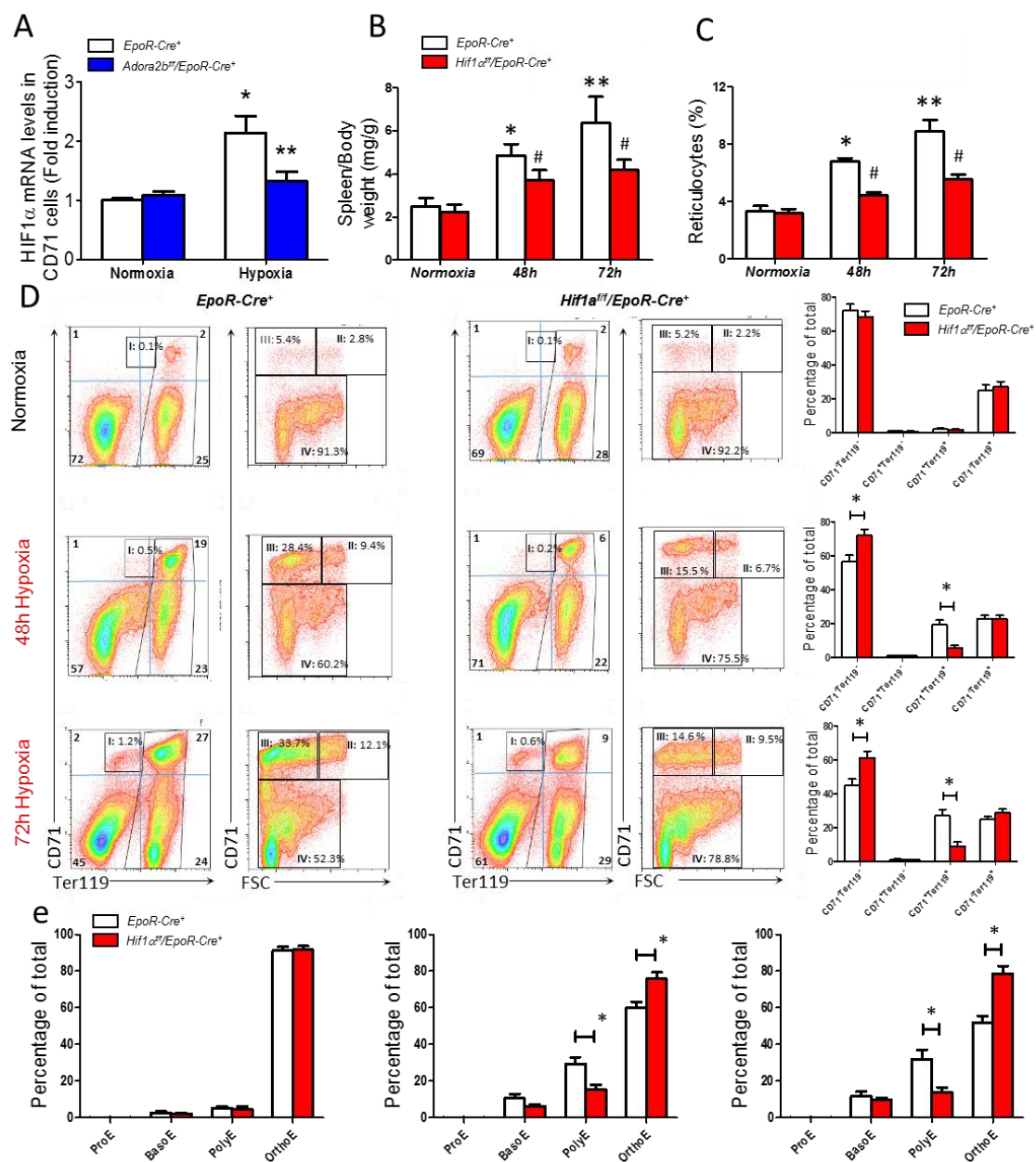


Figure. 28. Erythroid HIF-1 α contributes to hypoxia induced stress erythropoiesis (A) RT-PCR results showed that *Hif-1 α* mRNA is significantly induced in isolated CD71⁺Ter119⁺ splenic erythroids in *EpoR-Cre*⁺ mice under hypoxia, but not in *Adora2b^{fl/fl}EpoR-Cre*⁺ mice. (B-C) Hypoxia-induced stress erythropoiesis reflected by increased spleen size and percentage of reticulocyte in *EpoR-Cre*⁺ mice control mice, but was suppressed in *Hif-1 α ^{fl/fl}EpoR-Cre*⁺ mice. (D) Genetic deletion of erythroid HIF-1 α significantly reduced population II of CD71⁺Ter119⁺ proerythroblast progenitors in spleen compared to *EpoR-Cre*⁺ control mice after 72 hour hypoxia exposure. Data are expressed as mean \pm SEM; **P*<0.05 vs *EpoR-Cre*⁺ under normoxia; ***P*<0.05 *EpoR-Cre*⁺ vs *Hif-1 α ^{fl/fl}EpoR-Cre*⁺ mice under hypoxia (n=10).

3.3.5 Impaired commitment of erythroid versus myeloid lineage from hematopoietic stem cell in *Adora2b^{ff}EpoR-Cre⁺* mice in acute anemia model

Acute anemia is a life-threatening phenomenon when HCT levels drop dramatically in a short period of time, especially in stress conditions. To assess the clinical relevance of impaired stress erythropoiesis in *Adora2b^{-/-}* mice, we utilized a phenylhydrazine (PHZ)-induced hemolytic model, which is a well-accepted stress erythropoiesis model. 8-12 week old adult mice were consecutive injected with PHZ at day 0, 1 and 3 as previously, and erythropoietic parameters were detected at day 5. PHZ-treated *EpoR-Cre⁺* mice showed active erythropoiesis at the day 5 including recovered HCT levels, increased percentages of CD71⁺ and Ter119⁺ erythroid progenitors in bone marrow. However, impaired erythropoiesis was detected in PHZ-treated *Adora2b^{ff}EpoR-Cre⁺* mice. In contrast, suppressed myeloid lineage reflected by CD11b⁺ staining was detected in *EpoR-Cre⁺* mice, while enhanced levels in *Adora2b^{ff}EpoR-Cre⁺* mice after PHZ treatment (Fig. 29A-G). To further evaluate erythroid lineage differentiation, we assessed the Ter119⁺ cells on the basis of CD71 staining from stage I to stage IV. Intriguingly, PHZ-induced expansion of erythroid progenitors was induced the most in the stage II of proerythroblasts in *EpoR-Cre⁺* mice, but was suppressed in *Adora2b^{ff}EpoR-Cre⁺* mice (Fig. 30A).

3.3.6 Impaired commitment of erythroid versus myeloid lineage from hematopoietic stem cell in *Hif-1 α^{ff} EpoR-Cre⁺* mice in acute anemia model

To further evaluate the impact of HIF-1 α in acute anemia-induced stress erythropoiesis model, *EpoR-Cre⁺* mice and *Hif-1 α^{ff} EpoR-Cre⁺* mice were treated with consecutive injection of PHZ at day 0, 1 and 3 as previously, and erythropoietic parameters were detected at day 5. PHZ-treated *EpoR-Cre⁺* mice showed an active erythropoiesis at the day 5 including recovered their HCT levels, increased percentages of CD71⁺ and Ter119⁺ erythroid progenitors in bone marrow. However, impaired erythropoiesis was detected in PHZ-treated *Hif-1 α^{ff} EpoR-Cre⁺* mice. In contrast, suppressed percentage of myeloid lineage reflected by CD11b⁺ was detected in *EpoR-Cre⁺* mice, while enhanced levels in *Hif-1 α^{ff} EpoR-Cre⁺* mice after PHZ treatment (Fig. 31A-G). To further evaluate erythroid lineage differentiation, we assessed the Ter119⁺ cells on the basis of CD71 staining from stage I to stage IV. Intriguingly, PHZ-induced expansion of erythroid progenitors was induced the most in the stage II of proerythroblasts in *EpoR-Cre⁺* mice, but was suppressed in *Hif-1 α^{ff} EpoR-Cre⁺* mice (Fig. 32A).

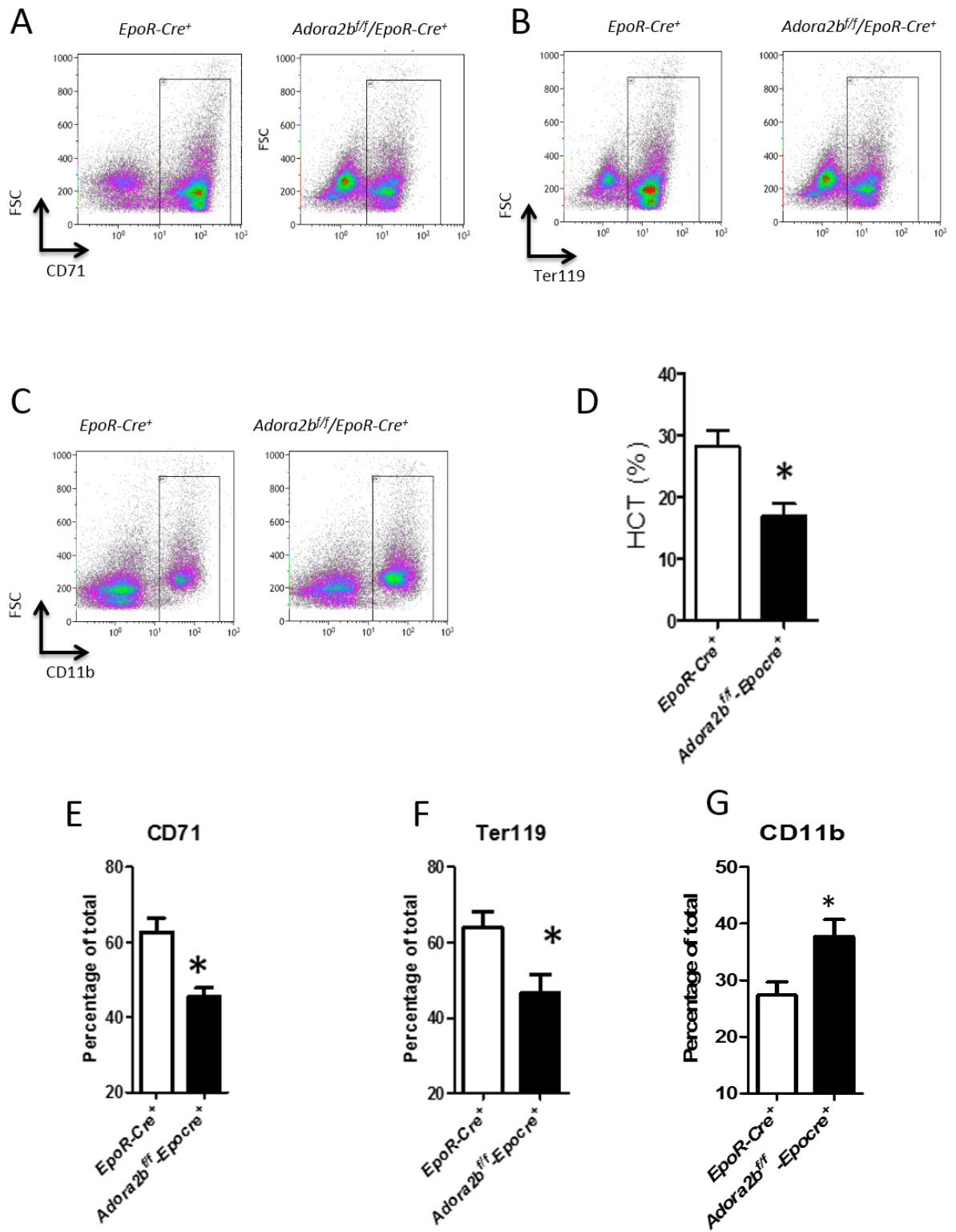


Figure. 29. Impaired commitment of erythroid versus myeloid lineage from hematopoietic stem cells in *Adora2b^{fl/fl}EpoR-Cre⁺* mice in acute anemia model

(A-G) Representative flow cytometry results showed that lower HCT level, suppressed percentages of CD71⁺ and Ter119⁺ erythroid progenitors, but higher CD11b myeloid progenitors in *Adora2b^{fl/fl}EpoR-Cre⁺* mice as compared to *EpoR-Cre⁺* mice after phenylhydrazine (PHZ) challenge. Data are expressed as mean \pm SEM; * $P < 0.05$ vs *EpoR-Cre⁺* after PHZ challenge (n=8).

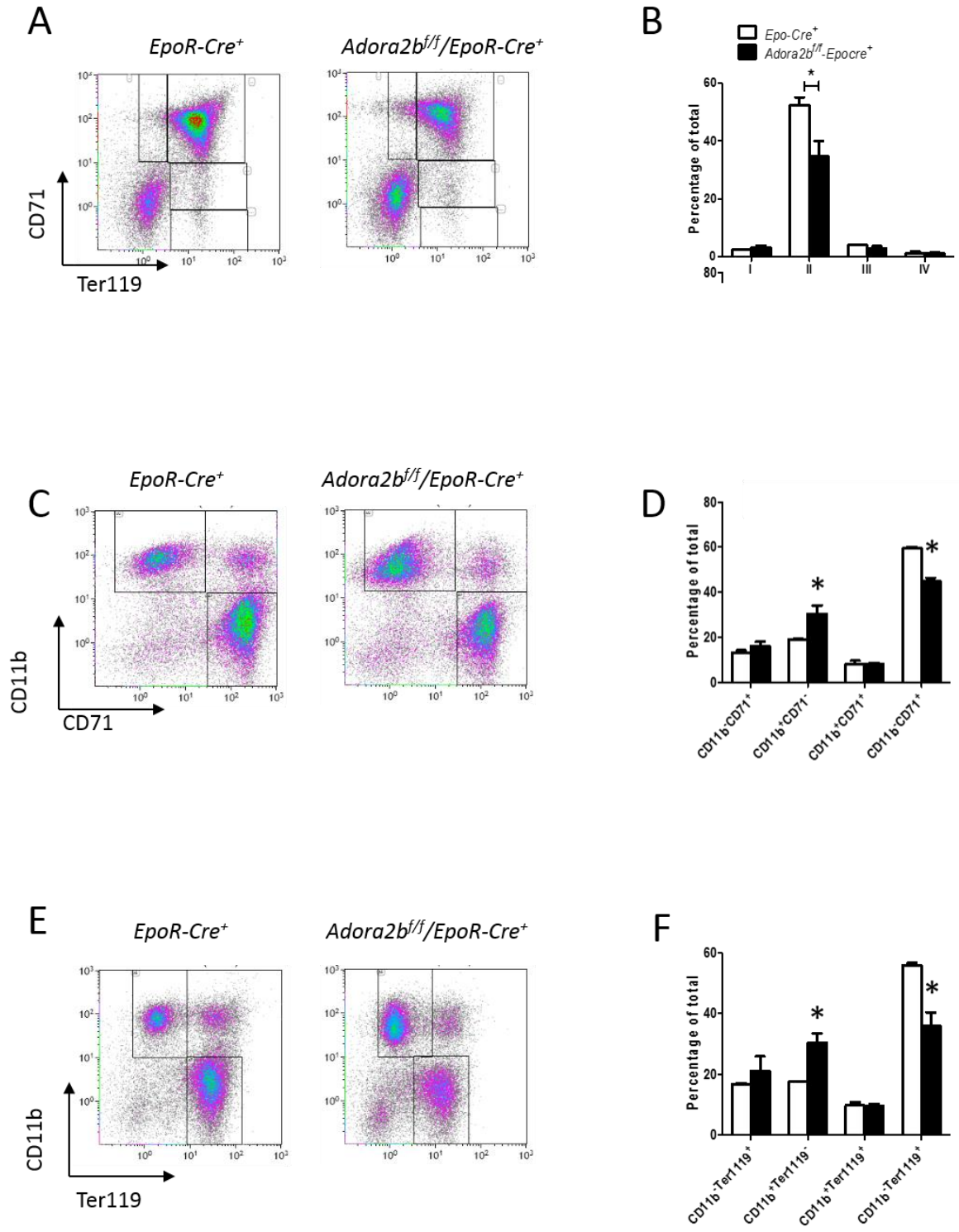


Figure. 30. Impaired commitment of erythroid versus myeloid lineage from hematopoietic stem cell in *Adora2b^{ff}EpoR-Cre⁺* mice after PHZ challenge

Erythroid lineage differentiation in *EpoR-Cre⁺* mice and *Adora2b^{ff}EpoR-Cre⁺* mice were assessed by flow cytometry by quantifying percentage of Ter119⁺ cells on the basis of CD71 staining from stage I to stage IV after PHZ challenge. Erythroid commitment in *EpoR-Cre⁺* mice and *Adora2b^{ff}EpoR-Cre⁺* mice were assessed by flow cytometry by quantifying percentage of erythroid lineage (CD71⁺CD11b⁻, Ter119⁺CD11b⁻), and myeloid lineage (CD71⁻CD11b⁺, Ter119⁻CD11b⁺) after PHZ challenge. Data are expressed as mean \pm SEM; * P <0.05 vs *EpoR-Cre⁺* after PHZ challenge. Data are expressed as mean \pm SEM; * P <0.05 vs *EpoR-Cre⁺* after PHZ challenge (n=8).

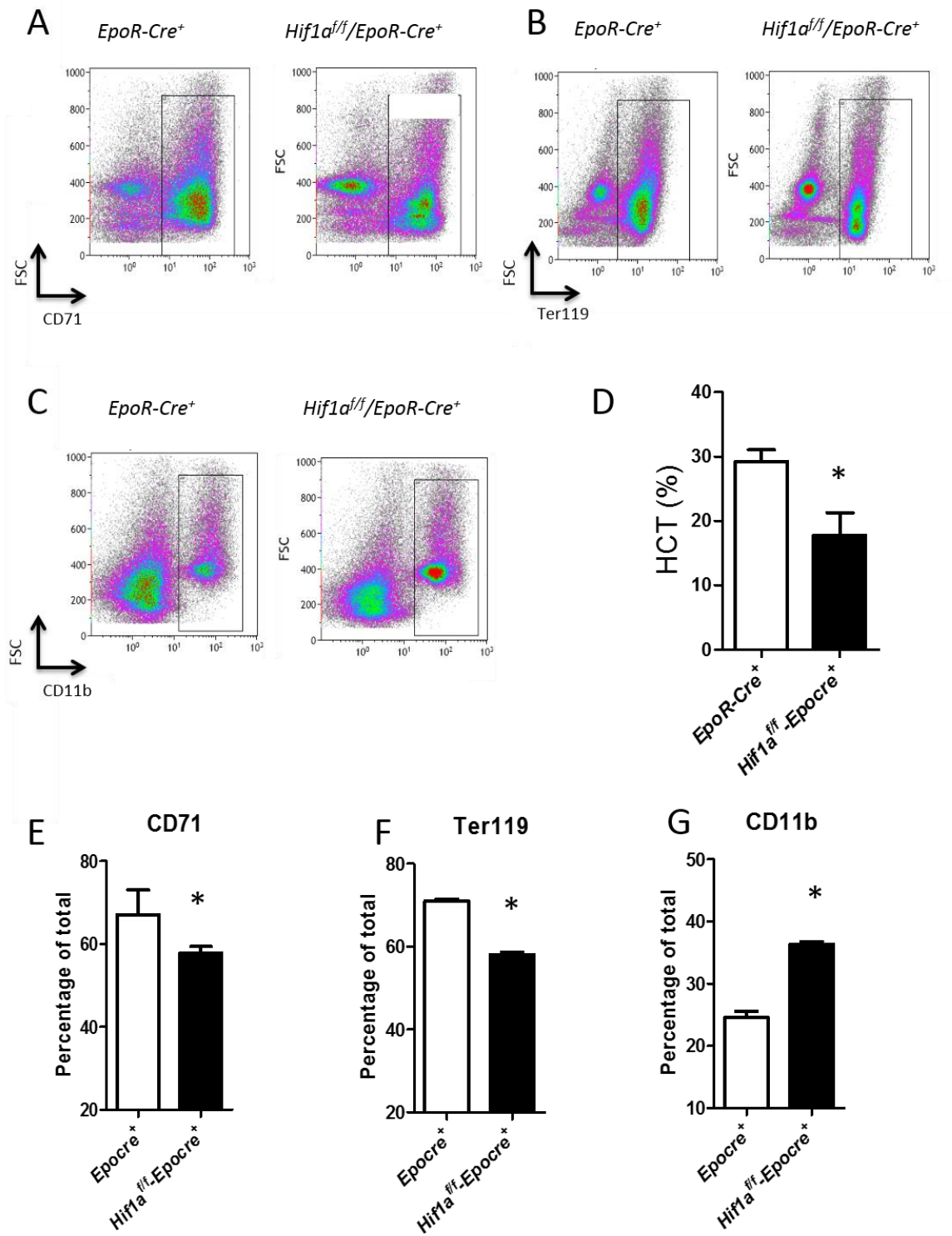


Figure. 31. Impaired commitment of erythroid versus myeloid lineage from hematopoietic stem cell in *Hif-1 α^{ff} EpoR-Cre⁺* mice in acute anemia model

(A-G) Representative flow cytometry results showed that lower HCT level, suppressed percentages of CD71⁺ and Ter119⁺ erythroid progenitors, but higher CD11b myeloid progenitors in *Hif-1 α^{ff} EpoR-Cre⁺* mice as compared to *EpoR-Cre⁺* mice after phenylhydrazine (PHZ) challenge. Data are expressed as mean \pm SEM; **P*<0.05 vs *EpoR-Cre⁺* after PHZ challenge (n=8).

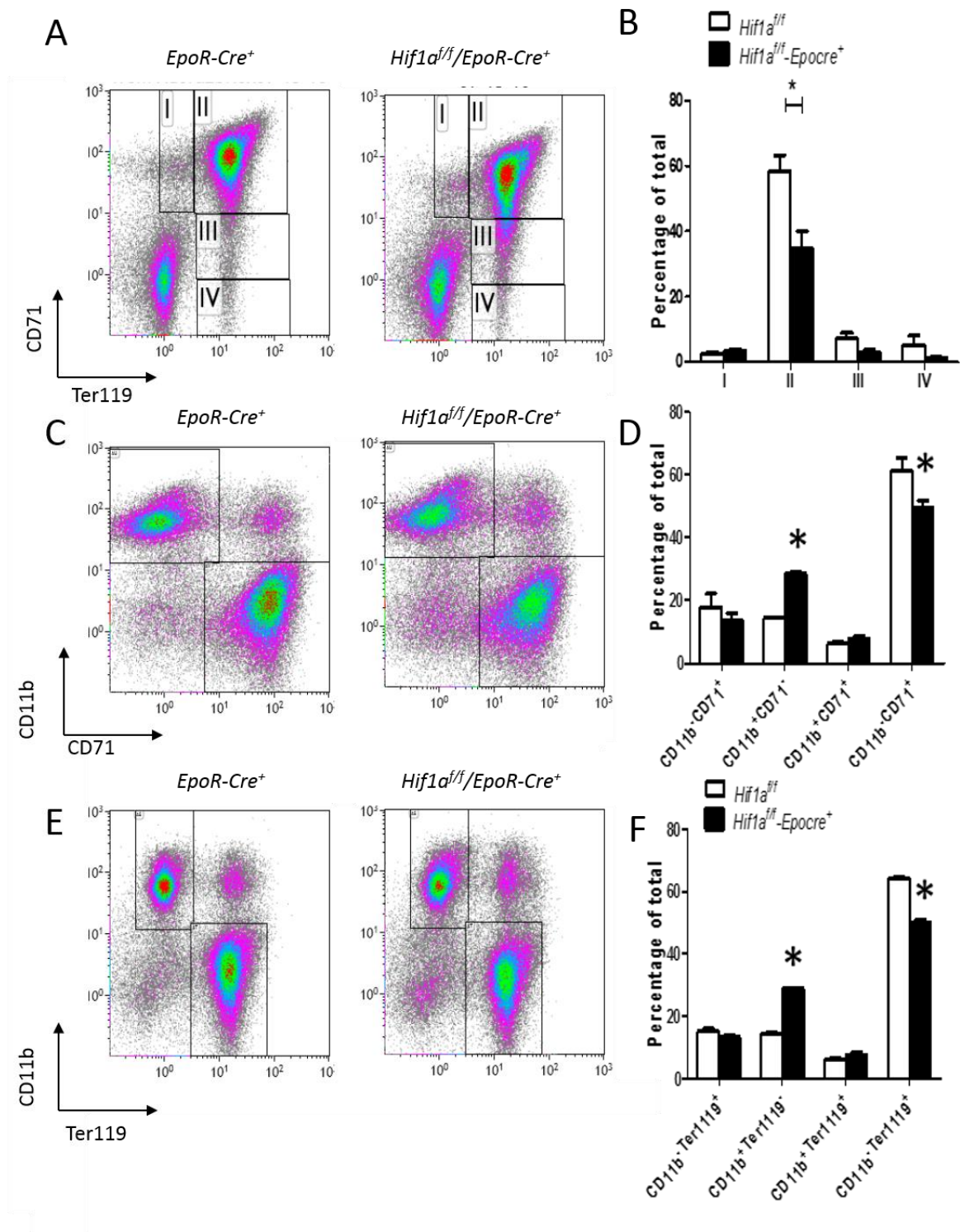
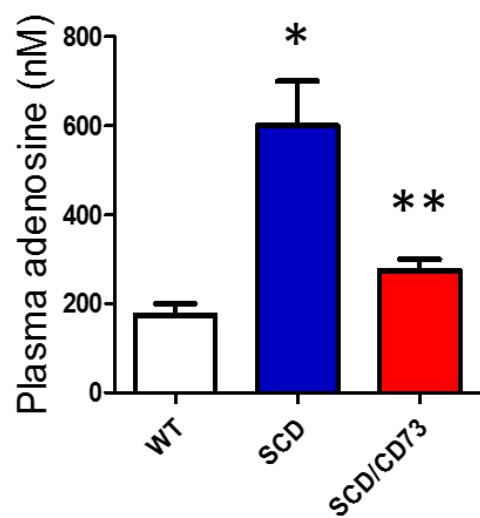


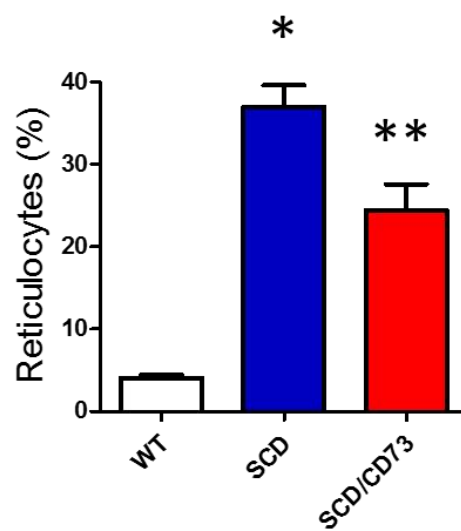
Figure. 32. Impaired commitment of erythroid versus myeloid lineage from hematopoietic stem cell in *Hif-1 α^{ff} EpoR-Cre⁺* mice after PHZ challenge

Erythroid lineage differentiation in *EpoR-Cre⁺* mice and *Hif-1 α^{ff} EpoR-Cre⁺* mice were assessed by flow cytometry by quantifying percentage of Ter119⁺ cells on the basis of CD71 staining from stage I to stage IV after PHZ challenge. Erythroid commitment in *EpoR-Cre⁺* mice and *Hif-1 α^{ff} EpoR-Cre⁺* mice were assessed by flow cytometry by quantifying percentage of erythroid lineage (CD71⁺CD11b⁻, Ter119⁺CD11b⁻), and myeloid lineage (CD71⁻CD11b⁺, Ter119⁻CD11b⁺) after PHZ challenge. Data are expressed as mean \pm SEM; **P*<0.05 vs *EpoR-Cre⁺* after PHZ challenge. Data are expressed as mean \pm SEM; **P*<0.05 vs *EpoR-Cre⁺* after PHZ challenge (n=8).

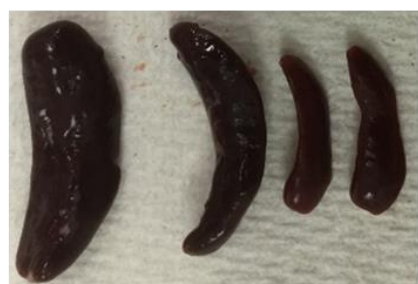
A



B



C



SCD

SCD/CD73

D

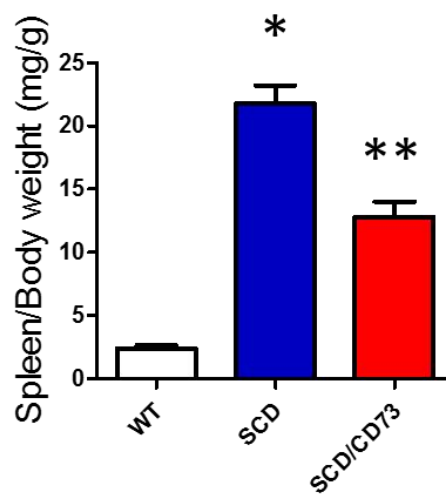


Figure. 33. CD73-mediated elevated circulating adenosine is responsible for increased stress erythropoiesis in SCD Tg mice

(A) Plasma adenosine were induced SCD Tg mice as compared to in WT mice, but genetic deletion of CD73 in SCD Tg mice background significantly attenuated plasma adenosine. (B-D) Splenomegaly and percentage of reticulocyte were significantly increased in SCD Tg mice as compared in WT mice, but genetic deletion of CD73 in SCD Tg mice background significantly attenuated increased spleen size and reticulocyte percentage. Data are expressed as mean \pm SEM; * P <0.05 vs WT mice; ** P <0.05 SCD/CD73 vs SCD (n=10).

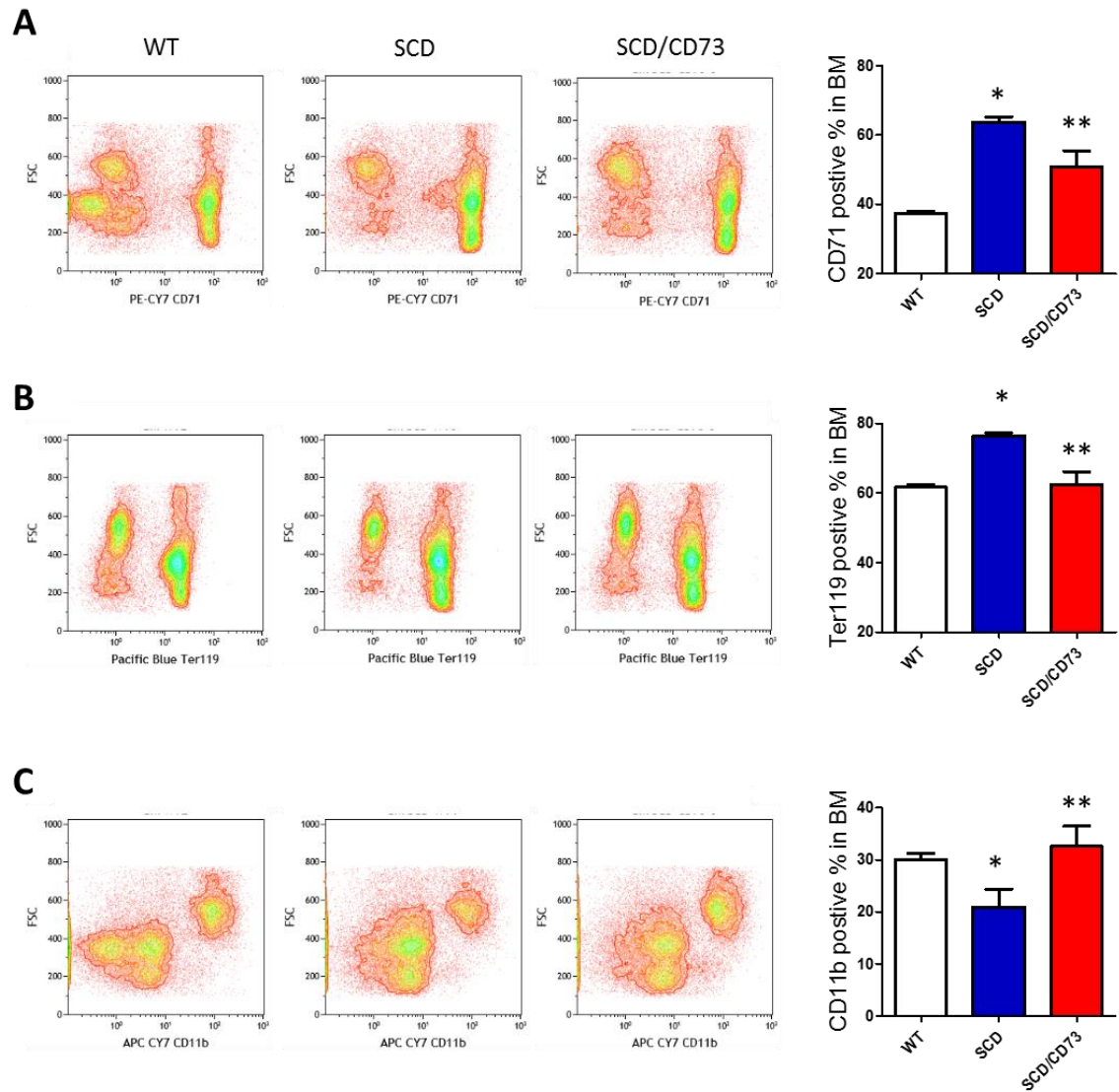


Figure. 34. Enhanced erythroid lineage versus myeloid lineage in SCD Tg mice

(A-C) Increased erythroid lineage reflected by percentage of splenic CD71⁺ and Ter119⁺ cells, and decreased myeloid lineage reflected by percentage splenic CD11b⁺ cells were detected by flow cytometry in SCD Tg mice but not in SCD/CD73 mice as compared to WT mice. Data are expressed as mean \pm SEM; * P <0.05 vs WT mice; ** P <0.05 SCD/CD73 vs SCD (n=10).

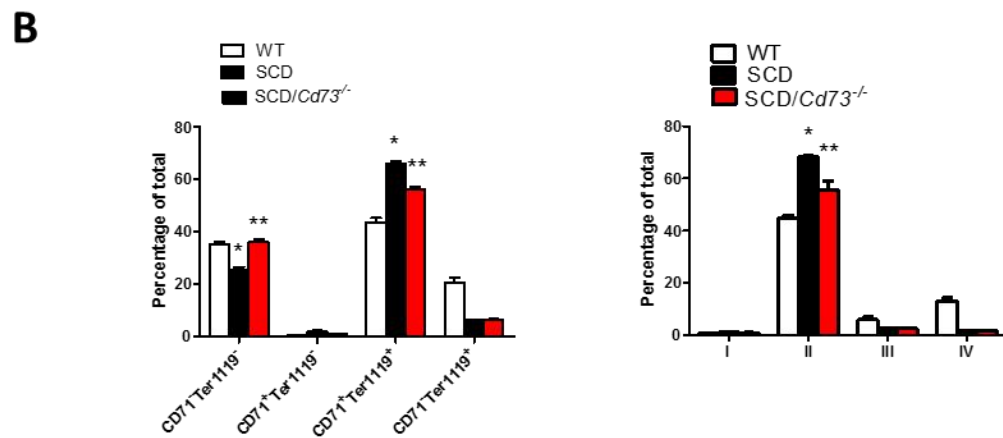
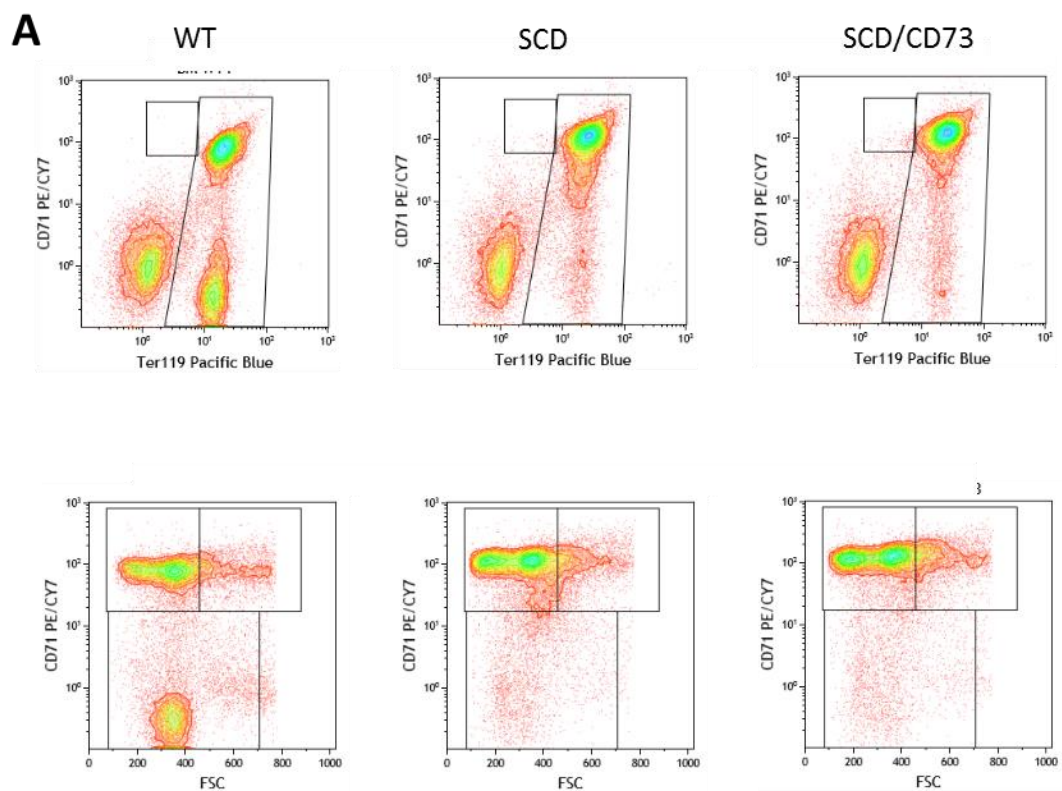


Figure. 35. (A-B) Erythroid lineage differentiation, reflected by assess the Ter119⁺ cells on the basis of CD71 staining from stage I to stage IV. Expansion of erythroid progenitors was induced the most in the stage II of erythroid progenitors in SCD Tg mice, but was suppressed in SCD/CD73 mice as compared to WT mice. Data are expressed as mean \pm SEM; * P <0.05 vs WT mice; ** P <0.05 SCD/CD73 vs SCD (n=10).

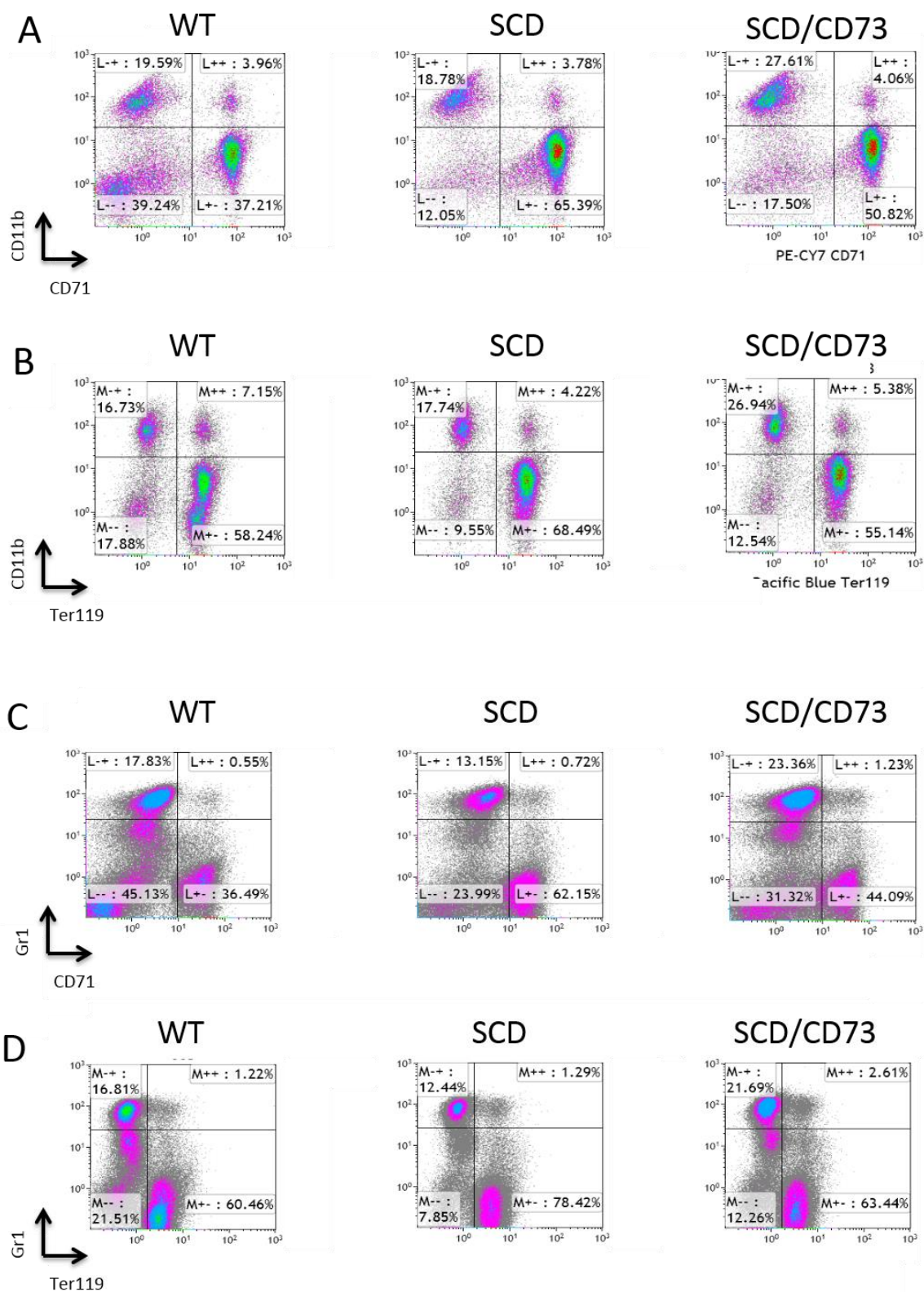


Figure. 36. Increased commitment of erythroid versus myeloid lineage from hematopoietic stem cell in SCD Tg mice in a CD73 dependent manner

(A-D) Erythroid commitment were assessed by flow cytometry by quantifying percentage of erythroid lineage (CD71⁺CD11b⁻, CD71⁺Gr1⁻, Ter119⁺CD11b⁻, Ter119⁺Gr1⁻), and myeloid lineage (CD71⁻CD11b⁺, CD71⁻Gr1⁺, Ter119⁻CD11b⁺, Ter119⁻Gr1⁺). Data are expressed as mean \pm SEM; * P <0.05 vs WT mice; ** P <0.05 SCD/CD73 vs SCD (n=10).

3.3.7 CD73-mediated elevation of plasma adenosine is responsible for stress erythropoiesis in humanized sickle cell disease mouse model

Sickle cell disease (SCD) is an inherited blood disorder that results from a mutation in hemoglobin, the protein that carries O₂ in erythrocytes throughout our bodies. The mutant hemoglobin causes the erythrocytes to acquire an unusual sickle shape that hinders movement through blood vessels. The clumps of sickled cells block blood flow resulting in pain, serious infections, and organ damage. It is widely accepted that SCD is a type of chronic hypoxia disease model due to the abnormal erythrocyte. Previous studies reported that stress erythropoiesis was occurred in SCD patients, and our current results showed that activated erythropoiesis was observed in SCD transgenic (Tg) mice. However, the molecular mechanism involved in activated erythropoiesis in SCD is unclear. Our previous studies showed that plasma adenosine is highly induced in both SCD patients and SCD Tg mice, and attenuation of circulating adenosine levels by PEG-ADA enzyme therapy significantly decreases erythropoietic parameters including spleen size and percentage of reticulocyte in SCD Tg mice, indicating that elevated plasma adenosine likely contributes to the stress erythropoiesis in SCD Tg mice⁹⁶. To test this hypothesis, we further deletion the CD73, a key enzyme generates adenosine extracellularly, in the background of SCD Tg mice. First, our HPLC results showed that plasma adenosine level was significantly attenuated in SCD/CD73 double knockout mice (Fig. 33A). Then, we showed that decreased plasma adenosine leads to suppressed percentage of reticulocyte and spleen size in SCD/CD73 double knockout mice compared to SCD Tg mice (Fig. 33B-D). Robust erythropoiesis was detected by flow cytometry in bone marrow reflected by

enhanced erythroid lineage including increased CD71⁺ population, Ter119⁺ population, and CD71⁺/Ter119⁺ population. However, activation of erythropoiesis was significantly attenuated in SCD/CD73 double knockout mice compared to SCD Tg mice. In addition, genetic deletion of CD73 in SCD Tg mice significantly attenuated elevation of stage II-CD71⁺Ter119⁺ erythroids compared to SCD Tg mice. Similar features were observed in the spleen (Fig. 34A-B and Fig. 35A-C). Intriguingly, enhanced myeloid lineage (CD71⁻CD11b⁺, CD71⁻Gr1⁺, Ter119⁻CD11b⁺, Ter119⁻Gr1⁺) was detected in SCD/CD73 double knockout mice as compared to SCD mice (Fig. 36A-D). Thus, this provides genetic evidence that adenosine signaling contributes to stress erythropoiesis by regulation of erythroid commitment of HSCs in SCD Tg mice.

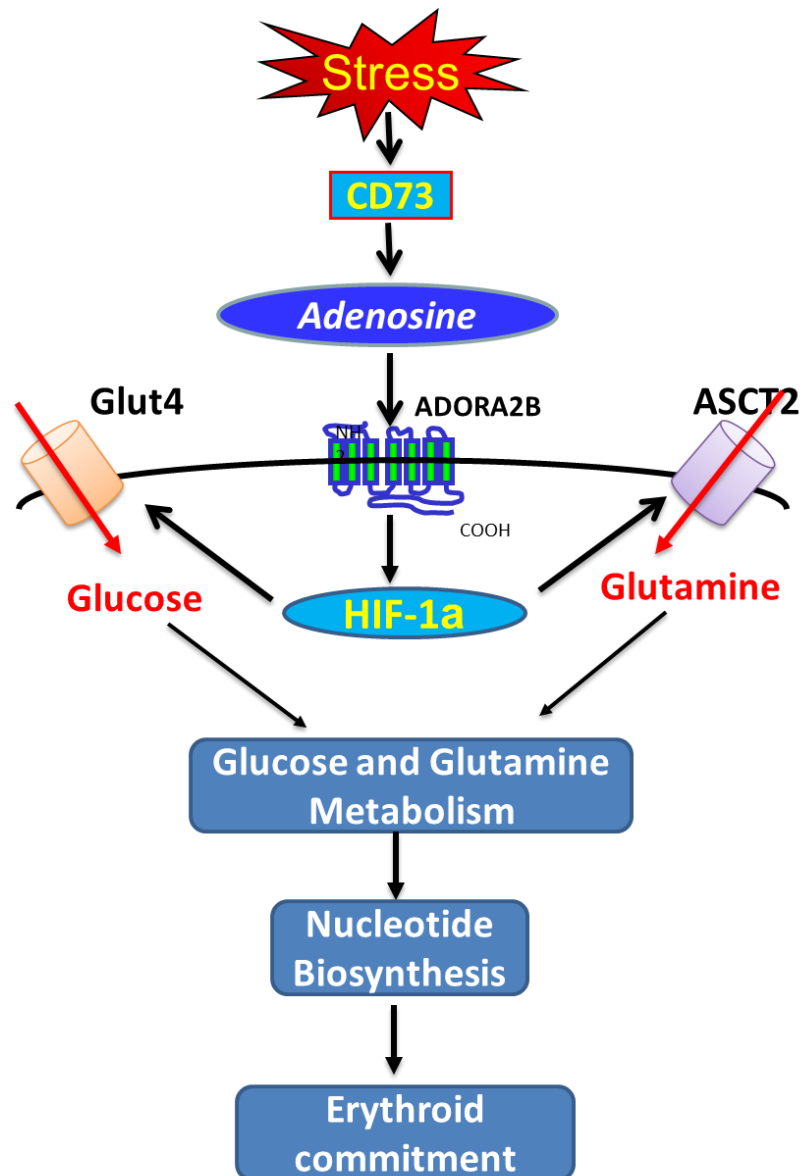


Figure 37. Working model of role of adenosine signaling in stress erythropoiesis

3.4 Discussion

Erythropoiesis, the formation of erythrocytes, is a dynamic process finely regulated by cytokines, hormones, and growth factors at transcriptional and translational levels^{134, 136}. Since erythrocytes, the most abundant cell type in the circulation, is the major source of oxygen to local tissues, erythropoiesis is stimulated under conditions of insufficient oxygen availability such as high altitude, blood loss, infection, and anemia. This so-called stress erythropoiesis process is defined by an expansion of the erythroid progenitor pool, associated with reticulocytosis and splenomegaly. Thus, stress erythropoiesis is an important stress adaptive response for survival. Although stress erythropoiesis has been linked with increased metabolic requirements, the molecular basis promoting metabolic reprogramming to enhance energy metabolism to support stress erythropoiesis remains unclear. Our recent studies identify a decisive role of elevated adenosine signaling in stress erythropoiesis. Mouse genetic studies showed that activation of adenosine signaling via erythroid ADORA2B promotes the commitment of erythroid both in spleen and bone marrow and in this way contributes to hypoxia-induced stress erythropoiesis independent of plasma Epo. Using unbiased high-throughput metabolic profiling coupled with microarray, we identified that erythroid ADORA2B contributes to an overall hypoxia metabolic reprogramming with substantially increased glycolysis, TCA cycle intermediates are produced by erythroid progenitors in mice. In addition, follow-up mouse anemia *in vivo* and mouse HSC *ex-vivo* studies showed that erythroid ADORA2B-HIF1 α is critical for erythroid commitment under stress condition. Thus, our studies identify adenosine ADORA2B is a previously unrecognized purinergic signaling underlying

hypoxia-induced erythropoiesis by regulation of metabolic reprogramming in erythroid progenitors, and likely discover new therapeutic targets to counteract hypoxia-induced injury by induction of erythrocyte number.

All living cells require energy for survival proliferation and differentiation to adapt to physiological and pathological stress conditions by metabolizing three major nutrients including glucose, glutamine and fatty acids. Changes in nutrient utilization and metabolism is termed "metabolic reprogramming" in response to any stimuli¹³⁷.

Although adenosine, HIF-1 α is induced by stress conditions such as hypoxia¹³⁸, the role and interaction of these hypoxic sensors in stress erythropoiesis were previously unrecognized. By using innovative unbiased high throughput metabolomics, coupled with our flux analysis of isotopically labeled metabolites, multiple genetic mouse models, we show for the first time that elevated adenosine signaling-mediated erythroid ADORA2B-HIF-1 α activation contributes to stress erythropoiesis. Our studies reported here allow us to identify a new pathway of adenosine signaling via erythroid ADORA2B-HIF-1 α regulating erythroid commitment, and subsequently contributing stress erythropoiesis by regulation of metabolic reprogramming. Our findings are novel and significant which identify multiple new therapeutic targets to regulate HSC lineage commitment, and thereby benefits for normal individuals at stress conditions and patients with hematology disorders.

Adenosine is ubiquitously existed in almost all of the cell type in our bodies, which is critical intermediate metabolite of nucleic acids as a building block. As a key signaling molecule, adenosine orchestrates the cellular response to hypoxia,

energy depletion, and stress challenge by activation of its G protein-coupled receptor (GPCR) on multiple cell types. Our results showed that ablation of erythroid ADORA2B in mice results in impaired erythropoiesis in response to stress, similar to CD73-deficient mice, indicating that adenosine-activated erythroid signaling pathway reveals new dimensions of the importance of adenosine signaling in adaptation to stress condition.

Although HIF-1 α is induced by stress condition, and play an important role in regulation of metabolic reprogramming¹³⁸, the role of HIF-1 α in stress erythropoiesis is not well characterized. HIF-1 α increases Adora2b gene expression under stress condition. More recently studies showed that ADORA2B feedback induces *Hif-1 α* gene expression in other cellular systems⁹⁴. By generating genetically modified mice with specific knockout in erythroid lineage, we showed that adenosine signaling via erythroid ADORA2B coupled with HIF-1 α reciprocal upregulation induces metabolic reprogramming, regulates erythroid commitment, and therefore promotes erythropoiesis in response to stress condition.

Taken together, our studies reveal novel pathway and molecules linking purinergic signaling with erythroid metabolic reprogramming and HIF-1 α targeted gene expression, which regulate erythroid commitment, and subsequently contributing stress erythropoiesis. The significance of our studies could be enormous since our results provide new insight to stress erythropoiesis for identification of innovative therapeutic approaches to promote erythropoiesis.

Chapter 4

4 Conclusion and future direction

4.1 Conclusion

Hypoxia is a condition in which the whole body or local organ and tissue is starved of sufficient oxygen supply. Hypoxia is a particularly dangerous condition for both normal individuals under high altitude hypoxia and patients with basic cardiovascular, respiratory and hemolytic diseases^{89, 90}. Due to a lack of fundamental understanding of the molecular mechanisms underlying adaptation to hypoxia, current strategies to counteract hypoxia are limited. As the only cell-type responsible for delivering O₂ to peripheral tissues in our body, mature RBCs quickly respond to hypoxia by increasing its O₂ delivery ability. Although it has been long speculated that RBC play an important adaptive role in response to hypoxia, specific molecules and signaling pathways involved in mature RBC "metabolic reprogramming" for rapid response to increase O₂ delivery to counteract hypoxia-induced tissue damage and prevent disease progression remain elusive.

By conducting pilot high altitude human studies, we observed that plasma adenosine concentration and sCD73 activity increased along with erythrocyte 2,3-BPG levels and O₂ releasing capacity in humans at high altitude. Follow-up mouse genetic studies demonstrated for the first time that CD73-dependent elevation of plasma adenosine signaling via erythrocyte specific ADORA2B is a key mechanism for hypoxia adaptation by inducing 2,3-BPG production and triggering O₂ release to counteract multiple tissue severe hypoxia. Mechanistically, we revealed that AMPK is a key enzyme that functions downstream of ADORA2B signaling and contributes to hypoxia-induced 2,3-BPG production and subsequent

O₂ release from erythrocytes in mice and humans. Overall, both human and mouse studies reported here provide strong evidence that CD73-dependent elevation of plasma adenosine, signaling via ADORA2B on erythrocytes, plays a beneficial role in preventing altitude-mediated tissue hypoxia by inducing 2,3-BPG production and triggering O₂ release in a AMPK-dependent manner (Fig. 6g). Thus, our findings reveal novel therapeutic targets to protect against tissue hypoxia and provide a strong foundation for future clinical trials. Our studies here addresses the challenge of identifying novel approaches for the safe and effective treatment to counteract acute hypoxia, a dangerous condition causing multiple tissue damage, dysfunction and sudden death. Our ultimate goal is to transform the results of our innovative metabolomic screen to new mechanism-based therapeutics to reduce hypoxia-induced tissue damage and dysfunction by targeting erythrocyte metabolites and their signaling pathways newly identified by our studies. Our research will likely provide new insight to our understanding of the molecular mechanisms of improving O₂ delivery from erythrocytes in any condition facing hypoxia. It may reduce morbidity and mortality for the billions individuals worldwide who suffer from hypoxia, which is beneficial for the health and well-being of billions of individuals with hypoxia.

Since it is impossible to analyze tissue damage at different time points in humans, we plan to conduct preclinical studies to address these questions in future direction. These studies are expected to provide evidence of the therapeutic effects of BAY-60-5683 (ADORA2B agonist), AICAR (AMPK agonist) and dipyridamole (adenosine booster) to promote O₂ release in human high altitude studies and reduce hypoxia. Furthermore, if we validate our mouse studies showing that

elevated adenosine-mediated activation of erythrocyte AMPK as we hypothesize, enhancing circulating adenosine levels by dipyridamole, ADORA2B by BAY-60-5683 and activation of AMPK by AICAR may represent novel effective therapeutic approaches for the treatment of hypoxia. We expect our human translational studies to provide a strong foundation of evidence to move forward quickly to conduct clinical trials in humans to prevent and treat hypoxia conditions including acute mountain sickness and many diseases associated with hypoxia.

Change of O₂ release capacity of erythrocytes is not the only adaptive mechanism in response to stress condition that contributes to enhancement of O₂ availability. Erythropoiesis, the production of erythrocyte number, is also one of the well-known adaptive mechanism in response to stress condition¹³⁰. Although adenosine is well known to be induced by hypoxia, the role and molecular basis of adenosine signaling in stress erythropoiesis were not previously recognized. Similarly, HIF-1 α is known to be induced by hypoxia¹³⁸. However, the role of HIF-1 α in hypoxia-induced stress erythropoiesis is not well characterized due to lack of genetically modified mice with specific knockouts of HIF-1 α in erythroid progenitors. With proof-of-principle novel genetic tools, our findings that elevated adenosine signaling through ADORA2B coupled with HIF-1 α reciprocal upregulation to induce hypoxic metabolic reprogramming to promote new RBC production to offset hypoxia and protect from hypoxic tissue damage. Using multiple innovative techniques including high throughput metabolomics analysis and mRNA microarray sequencing identify purinergic signaling with erythroid hypoxic metabolic reprogramming, coupled with HIF-1 α targeted gene expression, contributing to erythropoiesis by regulation of erythroid commitment.

Overall, our newly discovered molecular mechanisms underlying purinergic signaling with HIF-1 α in erythroid metabolic modulation identified by the study here will add a new chapter to erythroid physiology and pathology and open up new therapeutic possibilities to counter tissue hypoxia at all conditions.

Thus, our findings that elevated adenosine signaling through erythroid ADORA2B-HIF-1 α interactively feed forward to induce erythroid progenitor hypoxia metabolic reprogramming and stress erythropoiesis is extremely new and novel. Of significance, our studies identify new signaling pathway regulating erythroid metabolic modulation by a sophisticated and collaborative purinergic signaling network linked with HIF-1 α to promote erythroid commitment under stress erythropoiesis.

4.2 Future direction for chapter 2.

Our current studies showed that the activation of ADORA2B signaling is essential to stimulate 2,3-BPG production and promote O₂ release from RBCs to counteract tissue damage in mice in response to acute hypoxic exposure. Recently, our preliminary studies showing for the first time that genetic deletion of erythrocyte ADORA2B significantly reduced Ang II-induced RBC 2,3-BPG and P50 and in turn resulted in more kidney tissue damage featured with proteinuria compared to Ang II-infused WT mice. Thus, our study demonstrated the broad beneficial role of RBC ADORA2B-mediated elevated 2,3-BPG levels and O₂ delivery Ang II-induced kidney injury model. Additionally, we have recently made significant progress showing that AMPK functions downstream of ADORA2B and that AMPK activation by AICAR is sufficient to induce 2,3-BPG production and enhanced O₂ release in cultured human RBCs. These findings immediately suggest that promoting ADORA2B and AMPK activation are new therapeutic strategies to counteract hypoxia. Therefore, our finding of the beneficial role of elevated adenosine signaling via RBC ADORA2B coupled with AMPK activation to promote O₂ delivery highlights this signaling pathway as a therapeutic target to counteract tissue hypoxia.

Because of the importance of adenosine-mediated erythrocyte ADORA2B activation in counteracting tissue hypoxia in both hypoxia exposed and Ang II-infused models, we anticipate that these two drugs will reduce hypoxia or Ang II-induced tissue damage and dysfunction by rapidly inducing circulating adenosine, 2,3-BPG and O₂ release in these two independent models.

The beneficial role of RBC ADORA2B-mediated AMPK signaling in

physiological and pathological hypoxia-induced tissue damage had not been recognized until we conducted metabolomic screening and mouse genetic studies¹³⁹. Because hypoxia is frequently associated with CVD, enhancing O₂ release by these two new mechanistic treatments may also improve cardiovascular function by reducing tissue damage. Thus, we believe our mechanistic discoveries regarding erythrocyte ADOAR2B- mediated AMPK activation and subsequent O₂ release is highly innovative and extremely significant since these findings have added a significant new chapter to erythrocyte physiology and open up multiple new therapeutic opportunities for the treatment of acute high altitude sickness and hypoxia-related diseases.

4.3 Future direction for chapter 3.

To address what are the key enzymes and gene expression mediated by ADORA2B-HIF-1 α signaling network contributing to hypoxia metabolic reprogramming and supporting stress erythropoiesis, we will take advantage of multiple novel mouse genetic tools coupled with several innovative and cutting edge metabolic analytical techniques developed by us in recent years as below.

RNA deep sequencing: To determine if HIF-1 α as a key hypoxic transcription factor regulates other genes besides *Adora2b*, we will use robust RNA deep sequencing to compare the overall changes of RNA expression in CD71^{high}Ter119^{high} progenitors isolated from BM and spleen of *EpoR-Cre*⁺, *Adora2b*^{fl/fl}*EpoR-Cre*⁺, *HIF-1 α* ^{fl/fl}*EpoR-Cre*⁺ mice in response to hypoxia at different time points up to 72 hours. We will further use RT-PCR to validate and compare the gene expression levels of CD71^{high}Ter119^{high} progenitors isolated from BM and spleens of *EpoR-Cre*⁺, *Adora2b*^{fl/fl}*EpoR-Cre*⁺, *HIF-1 α* ^{fl/fl}*EpoR-Cre*⁺ mice under normoxia and hypoxia conditions at different time points up to 72 hours with a special focus on multiple known *HIF-1 α -targeted* genes including Glut1/4, pyruvate kinase and pyruvate dehydrogenase kinase.

Human in vitro studies: To validate our findings in mouse, after expansion human CD34⁺ BMCs, we will further culture the cells in erythroid differentiation medium with or without ADORA2B agonist (BAY-60-5683) or HIF-1 α stabilizer (DMOG).

In addition, we will further validate all of those identified HIF-1 α -targeted genes and metabolic changes from mice in humans by using the treated human

CD34⁺ HSC cultures above. Specifically, we will compare the effects of BAY-60-5683 or DMOG treatment on AMPK-mediated specific phosphorylation of the identified enzymes, gene expression and metabolites in those cells.

The proposed studies with multidisciplinary approaches have a high likelihood to identify multiple new pathways regulating erythroid metabolic modulation by a sophisticated and collaborative purinergic signaling network linked with HIF-1 α to promote erythroid commitment under stress erythropoiesis, and set up foundation to future clinical trials and provide therapeutic strategy to improve effective and sufficient erythropoiesis in various conditions.

Thus, my dissertation research has identified that adenosine signaling functions as novel a molecular mechanism involved in the regulation of erythrocyte O₂ release capacity and O₂ delivery capacity, providing therapeutic possibilities to enhance O₂ availability and prevent stress-induced tissue damage and inflammation.

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